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(54) Title: USE OF NERNSTEIN VOLTAGE SENSITIVE DYES IN MEASURING TRANSMEMBRANE VOLTAGE

(57) Abstract: Transmembrane potential measurement methods using cationic dyes, and anionic dyes are provided. Compositions of the cationic and anionic dyes and microfluidic systems which include the dyes and membranes are provided in conjunction with processing elements for transmembrane potential measurements.

> USE OF NERNSTEIN VOLTAGE SENSITIVE DYES IN MEASURING
> TRANSMEMBRANE VOLTAGE

CROSS REFERENCE TO RELATED APPLICATIONS

10   This application is a non-provisional of "USE OF NERNSTEIN
VOLTAGE SENSITIVE DYES IN MEASURING TRANSMEMBRANE
POTENTIAL," USSN 60/158,323, by Farinas and Wada, filed October 8, 1999, and
"USE OF NERNSTEIN VOLTAGE SENSITIVE DYES IN MEASURING
TRANSMEMBRANE POTENTIAL," USSN 60/168,792, by Farinas and Wada, filed
15   December 2, 1999, and "USE OF NERNSTEIN VOLTAGE SENSITIVE DYES IN
MEASURING TRANSMEMBRANE POTENTIAL," USSN 60/229,951, by Farinas and
Wada, filed September 1, 2000. *The present application claims priority to and benefit of
each of these prior applications, pursuant to 35 U.S.C. 119, as well as any other
applicable statute or rule.*

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FIELD OF THE INVENTION

The present invention is in the field of transmembrane potential
measurement using Nernstian dyes, e.g., in microfluidic systems.

BACKGROUND OF THE INVENTION

30   Cell-based assays are often preferred for an initial screening of
biologically active compounds, due to the approximation of in vivo systems by cells,
combined with their capability to be rapidly screened. A variety of cell responses to
stimuli can be detected, including cell death, transporter function and response to
chemical stimuli.

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The distribution of a permeable ion between the inside and outside of a cell or vesicle depends on the transmembrane potential of the cell membrane. In particular, for ions separated by a semi permeable membrane, the electrochemical potential difference ($\Delta\mu_j$) which exists across the membrane, is given by $\Delta\mu_j = 2.3$ RT log $[j_1]/[j_o]$ +zE_RF, where R is the universal gas constant, T is an absolute temperature of the composition, F is Faraday's constant in coulombs, $[j_1]$ is the concentration of an ion (j) on an internal or intracellular side of the at least one membrane, $[j_o]$ is the concentration of j on an external or extracellular side of the at least one membrane, z is a valence of j and E_R is a measured transmembrane potential. Thus, the calculated equilibrium potential difference (E_j) for ion j = $-2.3RT(zF)^{-1}\log[j_1]/[j_o]$ (this is often referred to as the Nernst equation). *See,* Selkurt, ed. (1984) <u>Physiology 5th Edition,</u> Chapters 1 and 2, Little, Brown, Boston, MA (ISBN 0-316-78038-3); Stryer (1995) <u>Biochemistry 4th edition</u> Chapters 11 and 12, W.H. Freeman and Company, NY (ISBN 0-7167-2009-4); Haugland (1996) <u>Handbook of Fluorescent Probes and Research Chemicals Sixth Edition</u> by Molecular Probes, Inc. (Eugene OR) Chapter 25 (Molecular Probes, 1996) and http://www.probes.com/handbook/sections/2300.html (Chapter 23 of the on-line 1999 version of the <u>Handbook of Fluorescent Probes and Research Chemicals Sixth Edition</u> by Molecular Probes, Inc.) (Molecular Probes, 1999) and Hille (1992) <u>Ionic Channels of Excitable Membranes,</u> second edition, Sinauer Associates Inc. Sunderland, MA (ISBN 0-87893-323-9) (Hille), for an introduction to transmembrane potential and the application of the Nernst equation to transmembrane potential. In addition to the Nernst equation, various calculations which factor in the membrane permeability of an ion, as well as Ohm's law, can be used to further refine the model of transmembrane potential difference, such as the "Goldman" or "constant field" equation and Gibbs-Donnan equilibrium. *See* Selkurt, ed. (1984) <u>Physiology 5th Edition,</u> Chapter 1, Little, Brown, Boston, MA (ISBN 0-316-78038-3) and Hille at e.g., chapters 10-13.

Increases and decreases in resting transmembrane potential — referred to as membrane depolarization and hyperpolarization, respectively — play a central role in many physiological processes, including nerve-impulse propagation, muscle contraction, cell signaling and ion-channel gating. Potentiometric optical probes (typically potentiometric dyes) provide a tool for measuring transmembrane potential and changes in transmembrane potential over time (e.g., transmembrane potential responses following

5   the addition of a composition which affects transmembrane potential) in membrane

containing structures such as organelles (including mitochondria and chloroplasts), cells

and in vitro membrane preparations. In conjunction with probe imaging techniques (e.g.,

visualization of the relevant dyes), these probes are employed to map variations in

transmembrane potential across excitable cells and perfused organs.

10   For example, the plasma membrane of a cell at rest typically has a

transmembrane potential of approximately -20 to -70 mV (negative inside) as a

consequence of K^+, Na^+ and Cl^- concentration gradients (and, to a lesser extent, H^+, Ca^{2+},

and HCO_3^-) that are maintained by active transport processes. Potentiometric probes are

important tools for studying these processes, as well as for visualizing, e.g., mitochondria

15   (which exhibit a large transmembrane potential of approximately -150 mV, negative

inside matrix), and for cell viability assessment. *See*, Molecular Probes (1996) chapter 25

and the references cited therein.

             Potentiometric probes include cationic or zwitterionic styryl dyes, cationic

rhodamines, anionic oxonols, hybrid oxonols and merocyanine 540. The class of dye

20   determines factors such as accumulation in cells, response mechanism and cell toxicity.

See, Molecular Probes 1999 and the reference cited therein; Plasek et al. (1996)

"Indicators of Transmembrane potential: a Survey of Different Approaches to Probe

Response Analysis." J Photochem Photobiol; Loew (1994) "Characterization of

Potentiometric Membrane Dyes." Adv Chem Ser 235, 151 (1994); Wu and Cohen (1993)

25   "Fast Multisite Optical Measurement of Transmembrane potential" Fluorescent and

Luminescent Probes for Biological Activity, Mason, Ed. , pp. 389-404; Loew (1993)

"Potentiometric Membrane Dyes." Fluorescent and Luminescent Probes for Biological

Activity, Mason, Ed. , pp. 150-160; Smith (1990) "Potential-Sensitive Molecular Probes

in Membranes of Bioenergetic Relevance." Biochim Biophys Acta 1016, 1; Gross and

30   Loew (1989) "Fluorescent Indicators of Transmembrane potential:

Microspectrofluorometry and Imaging." Meth Cell Biol 30, 193; Freedman and Novak

(1989) "Optical Measurement of Transmembrane potential in Cells, Organelles, and

Vesicles" Meth Enzymol 172, 102 (1989); Wilson and Chused (1985) "Lymphocyte

Transmembrane potential and Ca^{+2}-Sensitive Potassium Channels Described by Oxonol

35   Dye Fluorescence Measurements" Journal of Cellular Physiology 125:72-81; Epps et al.

(1993) "Characterization of the Steady State and Dynamic Fluorescence Properties of the

3

5   Potential Sensitive dye *bis*-(1.3-dibutylbarbituric acid) trimethine oxonol (DiBAC$_4$(3) in

model systems and cells" Chemistry of Physics and Lipids 69:137-150, and Tanner et al.

(1993) "Flow Cytometric Analysis of Altered Mononuclear Cell Transmembrane

potential Induced by Cyclosporin" Cytometry 14:59-69.

Potentiometric dyes are typically divided into at least two categories based

10   on their response mechanism. The first class of dyes, referred to as fast-response dyes

(e.g., styrylpyridinium dyes; *see*, e.g., Molecular Probes (1999) at Section 23.2), operate

by a change in the electronic structure of the dye, and consequently the fluorescence

properties of the dye, i.e., in response to a change in an electric field which surrounds the

dye. Optical response of these dyes is sufficiently fast to detect transient (millisecond)

15   potential changes in excitable cells, e.g., isolated neurons, cardiac cells, and even intact

brains. The magnitude of the potential-dependent fluorescence change is often small;

fast-response probes typically show a 2–10% fluorescence change per 100 mV.

The second class of dyes, referred to as slow-response or Nernstian dyes

(*See*, e.g., Molecular Probes, 1999 at Section 23.3), exhibit potential-dependent changes

20   in membrane distribution that are accompanied by a fluorescence change. The magnitude

of their optical responses is typically larger than that of fast-response probes. Slow-

response probes, which include cationic carbocyanines, rhodamines and anionic oxonols,

are suitable for detecting changes in a variety of transmembrane potentials of, e.g.,

nonexcitable cells caused by a variety of biological phenomena, such as respiratory

25   activity, ion channel permeability, drug binding and other factors. The structures of a

variety of available slow response dyes are found e.g., at table 25.3 of Molecular Probes

(1996).

Many slow, Nernstian dyes such as carbocyanines, rhodamines and

oxonols are used to measure transmembrane potential by virtue of voltage-dependent dye

30   redistribution and fluorescence changes resulting from the redistribution. Fluorescence

changes which may be caused by redistribution include: a change of the concentration of

the fluorophore within the cell or vesicle, a change in the dye fluorescence due to

aggregation or a change in dye fluorescence due to binding to intracellular or

intravesicular sites. Typically, 10-15 minutes of equilibration time is used to allow the

35   dyes to redistribute across the plasma membrane after changing the transmembrane

potential.

5   Despite the availability of transmembrane potential sensor compositions
and assays, there still exists a need for additional classes of dyes and for new assays and
techniques for using potentiometric dyes in biological assays. The present invention
fulfills these and a variety of other needs which will become apparent upon complete
review of the following.

10   **SUMMARY OF THE INVENTION**
It is surprisingly discovered that membrane permeable cationic nucleic
acid staining dyes can be used as potentiometric dyes for measuring changes in
transmembrane potential. In addition, it was discovered that using both cationic dyes
(including, but not limited to membrane permeable cationic nucleic acid staining dyes)
15   and anionic membrane permeable redistributing dyes for monitoring changes in
transmembrane potential increases the dynamic range and sensitivity of transmembrane
potential measurements. Compositions comprising these two classes of dyes and a
membrane, as well as microfluidic systems for using the dyes to measure transmembrane
potential, are provided. Further it was discovered that measuring the time course of dye
20   uptake, rather than equilibrium distributions of the dyes, leads to improvements in signal
to noise ratio, speed of the assay and other benefits.

Accordingly, the present invention provides methods of generating optical
signals which depend on transmembrane potential or one or more change in
transmembrane potential. For example, in one class of embodiments, the methods
25   include providing a first component comprising one or more membrane, adding a cationic
membrane permeable nucleic acid staining dye to the first component, and monitoring a
first signal output from the cationic membrane permeable nucleic acid staining dye. To
monitor changes in transmembrane potential, changes in the first signal output are
monitored over time. The first signal output is then correlated with the transmembrane
30   potential to provide an indication of transmembrane potential or changes in
transmembrane potential. Typically, the first composition is also contacted with an
anionic membrane permeable redistributing dye to increase the sensitivity and dynamic
range of the assay.

In one common format, the relevant components are provided in a
35   microfluidic system. For example, a method of producing a signal which is dependent on
transmembrane potential is provided, in which a first mixture which includes one or more

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5   membranes and one or more voltage sensitive dyes is flowed through a first channel

region. At least a first signal output is monitored from at least one of the voltage sensitive

dyes, thereby producing a signal which is dependent on the transmembrane potential

across the one or more membranes. For example, the voltage sensitive dyes can include

one or more membrane permeable redistributing dyes, including one or more ionic dye.

10   The one or more membrane permeable dyes are typically flowed from a source to the first

channel region and into contact with the one or more membranes and flow of the

membrane permeable labels across the membrane is detected by monitoring the one or

more signal outputs from the membrane permeable labels, typically before equilibrium is

reached. The mixture can include a cationic dye, a cationic membrane permeable nucleic

15   acid staining dye, an anionic dye and/or a neutral dye. The one or more voltage sensitive

dyes can include, e.g., an anionic or cationic dye (or both), including any or all of:

Oxonol V, Oxonol VI, DiBAC4(3), DiBAC4(5), DiBAC2(3), a cationic dye, a cationic

membrane permeable nucleic acid staining dye, and a SYTO dye such as SYTO 62.

For example, a cationic dye, such as the cationic membrane permeable

20   nucleic acid staining dye, and the first component are flowed through at least a first

microfluidic channel comprising flowing the cationic membrane permeable nucleic acid

staining dye through the first or second microchannel and into contact with the at least

one membrane. An anionic membrane permeable redistributing dye can also be flowed

through the first or second channel and into contact with the at least one membrane.

25   Thus, in microfluidic formats, methods of measuring or monitoring

changes in a transmembrane potential are provided. In the methods, a first component

which includes one or more membrane is flowed from a source to a first channel region.

A labeling composition comprising a membrane permeable label is flowed into contact

with the membrane. The membrane is altered in some way that causes an alteration in

30   transmembrane potential, e.g., by changing the ionic composition on one side of the

membrane (e.g., inside or outside of a cell) or by changing the permeability of the

membrane to ions. The flow of the membrane permeable label across the membrane is

monitored by monitoring a first signal output from the membrane permeable label,

thereby measuring changes in the transmembrane potential.

35   The above methods can include contacting the membrane to one or more

transmembrane potential modulatory compositions and monitoring an effect of the one or

5   more transmembrane potential modulatory compositions on the transmembrane potential
(e.g., by monitoring the first signal), thereby monitoring an effect of the one or more
transmembrane potential modulatory compositions on the transmembrane potential. This
can be used as a drug screening method for testing potential modulatory compounds for a
transmembrane potential modulatory activity. Examples of modulatory compositions

10   include hyperpolarization buffers, depolarization buffers, compounds which alter the
ionic permeability of a membrane, and the like. In addition, control modulators
(modulators having a known effect on transmembrane potential in the relevant assay) can
be compared to test modulators having unknown effects to determine membrane
modulatory activity of the test modulators. Dose response curves for either control or test

15   modulators can be determined and the curves compared.

Control and test modulators can affect, e.g., transporter activity, ion
channel activity, or other factors which have an effect on transmembrane potential and
changes in transmembrane potential. Examples of test and control modulators include a
variety of compounds which effect membrane ionic permeability, ionic potential or the

20   like, including neurotoxins (e.g., such as palytoxin), sets of neurotoxins,
neurotransmitters, sets of neurotransmitters, proteins, sets of proteins, peptides, sets of
peptides, lipids, sets of lipids, carbohydrates, sets of carbohydrates, organic molecules,
sets of organic molecules, drugs, sets of drugs, receptor ligands, sets of receptor ligands,
antibodies, set of antibodies, cytokines, sets of cytokines, chemokines, sets of

25   chemokines, hormones, sets of hormones, cells, sets of cells and the like.

In general, the time course of dye translocation across the membrane
depends on the transmembrane potential across the membrane. Thus, at a selected time
(t) after adding a dye to a membrane, the amount of signal from the dye is correlated to
transmembrane potentials. Typically, (t) can be less than about 100 seconds. Commonly,

30   (t) is between about 0.1 and 80 seconds, e.g., between about 10 and 70 seconds. A ratio
of first and second signals from the cationic and anionic dyes noted above (e.g., over
time) can be determined to further refine estimates of changes in transmembrane
potential.

Examples of useful dyes include cyclic-substituted unsymmetrical cyanine

35   dyes and other cationic membrane permeable nucleic acid stains. Examples of useful
dyes include Blue-fluorescent SYTO dyes, Green-fluorescent SYTO Dyes, Orange-

5   fluorescent SYTO dyes, Red-fluorescent SYTO dyes such as SYTO 62, Pur-1, thiazol,

aryl, 2DS-7J1, Hoechst 33258, Hoechst 33342 and hexidium iodide. Common anionic

membrane permeable redistributing dyes include anionic bis-isoxazolone oxonol dyes,

bis-oxonol dyes and others. For example, the anionic membrane permeable redistributing

dye can be e.g., Oxonol V, Oxonol VI, $DiBAC_4(3)$, $DiBAC_4(5)$ and/or $DiBAC_2(3)$.

10   Example dye concentrations in the relevant systems are typically between about 0.01 and

about 50 μM. For example, the cationic dye can be SYTO 62, added to the first

component to a concentration of between about 0.01 and about 50 μM and the anionic

dye can be DiBAC4(3), added to the first or second component at a concentration of

between about 0.01 and about 50μM.

15   As noted, examples of membranes of interest include cells, organelles,

artificial membranes, membrane preparations of artificial or naturally occurring

membrane sources, and the like. Membrane preparations can be suspended in any suitable

buffer, e.g., a fluid comprising a membrane permeable ion such as Na^+, K^+, Cl^-, H^+, Ca^{2+},

or HCO_3^-. In one embodiment, the membrane is present in an intact or live cell such as an

20   animal cell, a plant cell, a fungal cell, a bacterial cell, or the like. For example, the cell

can be a mammalian cell such as a primate cell, a rodent cell, a canine cell, a feline cell,

or a livestock cell, or can be e.g., an insect cell or other animal cell. The cell can be a

cultured cell such as a THP-1 cell, a COS cell, a CHO cell, a HEK cell, a jurkat cell, a

βRL cell, a HeLA cell, an NIH 3T3 cell, an RBL-2H3 cell, or the like. The cell can also

25   be a primary cell such as a cell isolated from endoderm, ectoderm, mesoderm,

differentiated tissue, undifferentiated tissue, partially differentiated tissue, blood,

peripheral blood, nerve, muscle, skin, bone, or the like.

           Typically, signal outputs from dyes are detected by monitoring one or

more fluorescent emission produced by the relevant dye. This can be performed

30   spectrophotometrically, optically or, e.g., via microscopy.

           In one aspect, the invention provides a microfluidic device for monitoring

transmembrane potential. The microfluidic device includes a body structure having at

least one microscale cavity (e.g., microchannel, microchamber, or the like) disposed

therein. A target source of a first composition which includes at least one membrane is

35   fluidly coupled to the at least one microscale cavity (e.g., a microscale channel, chamber,

well, column or the like). A cationic membrane permeable staining dye source which

5   includes one or more cationic membrane permeable nucleic acid staining dye, is fluidly coupled to the at least one microscale cavity. Alternatively or in addition, an anionic membrane permeable redistributing dye source which includes one or more anionic redistributing dye is fluidly coupled to the at least one microscale cavity. During operation of the device, the first composition is contacted, in the presence of the cationic

10   membrane permeable staining dye, and/or the anionic membrane permeable redistributing dye, to at least one transmembrane potential modulatory composition.

          In applications where the device is used for screening effects of modulatory compositions, the device can include a source of at least one potential membrane modulatory composition fluidly coupled to the at least one microscale cavity.

15   The potential membrane modulatory composition can be, e.g., a membrane hyperpolarization buffer, a membrane depolarization buffer, or a compound which alters ionic permeability of the membrane.

          The device typically includes a signal detector located proximal to the microscale cavity. The signal detector detects the detectable signal, e.g., for a selected

20   length of time (t). For example, the detector can include a spectrophotometer, or an optical detection element. Commonly, the signal detector is operably coupled to a computer, which deconvolves the detectable signal to provide an indication of the transmembrane potential, e.g., an indication of a change in the potential over time.

          In one typical embodiment, during operation of the device, the first

25   composition comprising at least one membrane is flowed from the target source into the cavity, e.g., into a microchannel. The potential membrane modulatory composition is flowed from the target source into contact with the first composition. The cationic membrane permeable staining dye and/or an anionic membrane permeable redistributing dye is flowed into contact with the first composition and the detectable signal is

30   monitored at a selected time (t) after contact of the first composition with the cationic membrane permeable staining dye and/or the an anionic membrane permeable redistributing dye.

          In one aspect, the invention provides a composition, e.g., for practicing the methods noted above. The composition includes a first component comprising a

35   membrane, a cationic membrane permeable nucleic acid staining dye, and an anionic membrane permeable redistributing dye. The membrane component can include, e.g., a

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5   cell, mitochondria, chloroplast, cell vesicle, a membrane preparation of a cell or cell

component, or an artificial membrane. The cell can be an intact cell which can be, e.g.,

an animal cell, a plant cell, a fungal cell or a bacterial cell. For example, the cell can be

any of those noted herein.

Similarly, the cationic membrane permeable nucleic acid staining dye and

10   the anionic membrane permeable redistributing dye can be any of those noted above with

reference to the methods of the invention. The composition can also include buffers,

ions, etc., as noted herein. A container or microfluidic processor comprising the

composition is also a feature of the invention. For example, the composition of the

invention can be present in a kit or microfluidic processor. The kit can additionally

15   include, e.g., instructions for practicing the method of the invention, control compounds,

test compounds, containers for holding reagents, packaging materials, or the like. ·

BRIEF DESCRIPTION ON THE FIGURES

Figure 1 is a line graph showing the time course of dye uptake as a

function of transmembrane potential. Line A shows resting potential, high sodium. Line

20   B shows depolarized, high potassium.

Figure 2 is a line graph showing change in SYTO 62 fluorescence over

time. Line A shows resting potential, high sodium. Line B shows depolarized, high

potassium.

Figure 3, panel A is a bar graph showing fluorescence ratio of $DiBAC_4(3)$

25   to SYTO® 62. Panel B shows two graphs of fraction of cells vs. $DiBAC_4(3)$ intensity

and SYTO 62 intensity. Line A shows resting cells in high sodium buffer, line B shows

depolarized cells in UTP/high potassium buffer and line C shows hyperpolarized cells in

UTP/high sodium buffer.

Figure 4 is a bar graph of a dose-response curve.

30   Figure 5 is a schematic of the Caliper® 3B5 microfluidic processor.

Figure 6 is a schematic of an integrated system of the invention.

Figure 7 is a schematic showing further details of an integrated system of

the invention.

Figure 8 is a schematic showing further details of an integrated system of

35   the invention.

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5   Figure 9, panels 1-D schematically depicts a membrane potential assay and

shows data from the assays.

Figure 10, panels A-C schematically shows depolarization and

hyperpolarization assays.

Figure 11 is a data figure schematically showing, e.g., low cell

10   consumption and high data quality during use of primary cells in assays of the invention.

Figure 12 is a calibration curve showing the ratio of dibac/syto 62 versus

membrane potential.

DETAILED DESCRIPTION OF THE INVENTION

The distribution of a permeable ion between the inside and outside of a

15   cell or vesicle, or between the inner and outer leaflet of a membrane, depends on

transmembrane potential. The voltage dependence of the distribution is essentially

governed by the Nernst equation. Many slow Nernstian dyes such as carbocyanines,

rhodamines and oxonols are used to measure transmembrane potential by virtue of

voltage-dependent dye redistribution and fluorescence changes resulting from the

20   redistribution. Fluorescence changes which are caused by redistribution include: a

change of the concentration of the fluorophore within the cell or vesicle, a change in the

dye fluorescence due to aggregation or a change in dye fluorescence due to binding to

intracellular or intravesicular solutes. Typically, 10-15 minutes of equilibration time is

used to allow the dyes to redistribute across the plasma membrane after changing the

25   transmembrane potential (TMP).

The present invention concerns a class of dyes which are newly discovered

to be suitable for generating optical signals which depend on TMP. The class of dyes are

cationic, membrane permeable, nucleic acid stains such as SYTO 62. The invention also

relates to the method in which they are used to measure changes in TMP. After addition

30   of SYTO 62 to cell suspensions, a time course of SYTO 62 fluorescence was found to be

dependent on the transmembrane potential across the cell plasma membrane. A

significant advantage of this new class of probes compared to other cationic probes is that

a large fraction of the fluorescence change arises from fluorophore located outside of

mitochondria. The electromotive force on these cationic dyes is also counter to anionic

35   dyes, leading to important advantages when the two types of dyes are used together in

TMP assays.

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5   A preferred method for using this class of dyes is to use the new class of

dyes together with a traditional anionic Nernstian dye such as DiBAC$_4$(3) and to measure

the ratio of their fluorescence intensities. This approach has several advantages, such as a

higher signal to noise ratio than when the dyes are used separately. Another advantage is

the ability to detect voltage changes involving both hyperpolarization and depolarization

10   more efficiently, because depolarization enhances anionic dye fluorescence (decreasing

cationic dye fluorescence) and hyperpolarization enhances cationic dye fluorescence

(decreasing anionic dye fluorescence).

           A further preferred method is to measure cell-associated fluorescence of

the dyes after contacting a cell with the dyes, but before an equilibrium distribution of the

15   dyes has been established. This kinetic approach increases sensitivity, provides larger

dynamic range to the assay, and allows measurements to be made more quickly at a given

sensitivity level. This approach has operational advantages such as eliminating manual

preload of cells with dyes that can be a source of "run to run" and "within run" assay

variation or cellular toxicity. This method is also highly suited to microfluidic processor

20   formats that allow an automated addition of test samples and dyes, followed by a brief

incubation and reading of one or two color fluorescence.

           The specific chemistry of the cationic nucleic acid (e.g., DNA and/or

RNA) stain can be varied, as can the specific chemistry of the anionic Nernstian dye. The

methods can utilize microfluidic instrumentation or more traditional assay formats such

25   as fluorescence microplate readers with multiple reagent addition capability.

DEFINITIONS

           A "transmembrane potential" is the work needed to move a unit of charge

across a membrane.

           A "cationic membrane permeable nucleic acid staining dye" is a dye which

30   has a positive charge under specified pH (e.g., physiological pH) and which binds to or

otherwise associates with a nucleic acid, where the dye can cross a selected membrane

such as the membrane of an intact cell.

           An "anionic Nernstian dye" or an anionic membrane permeable

redistributing dye is a dye which has a negative charge at a specified pH (e.g.,

35   physiological pH) and which is membrane permeable and whose distribution between the

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5   inside and outside of the space bounded by the membrane or between the inside and
       outside of the membrane, depends on the transmembrane potential across the membrane.

               A "neutral dye" has an overall neutral charge under the relevant conditions
       at issue, e.g., a specified pH (e.g., physiological pH).

               A "voltage sensing composition" is a transmembrane potential indicator

10   comprising a fluorescent dye. Common voltage sensing compositions of the invention
       include one or more cationic membrane permeable nucleic acid staining dye(s), or,
       optionally, one or more additional cationic potentiometric dye, and, optionally, an anionic
       membrane permeable redistributing dye.

               A membrane is "depolarized" when the transmembrane potential across

15   the membrane is zero. A membrane is "hyperpolarized" when the transmembrane
       potential is more negative than the resting potential of the membrane.

               A membrane is "permeable" to a given component (dye, ion, etc.) when
       that component can equilibrate across the membrane in about 24 hours or less, and
       generally within about 12, 5, 1 or 0.5 hours, or less. Permeability can be dependent upon

20   the relevant conditions, e.g., temperature, ionic conditions, voltage potentials, or the like.

MEMBRANE PERMEABLE CATIONIC DYES

               As noted, preferred voltage sensing compositions of the invention include
       any of a variety of cationic membrane permeable nucleic acid staining dyes. Such dyes
       are available and can be used in the assays, compositions and devices of the invention.

25   A feature of the invention is the discovery that cell-permeant nucleic acid
       stains are suitable for use in potentiometric TMP measurements. For example, cyanine
       nucleic acid-staining SYTO dyes available from Molecular Probes (See, e.g., Molecular
       Probes 1999, Chapter 8). These dyes are generally suitable for use in potentiometric
       TMP measurements. These include the SYTO orange fluorescent nucleic acid stains

30   (e.g., SYTO 80-85); the SYTO blue fluorescent nucleic acid stains (SYTO 40-45); the
       SYTO green fluorescent nucleic acid stains (SYTO 11-16, 18, and 20-25) and the SYTO
       red fluorescent Nucleic Acid Stains (SYTO 17, and 59-64). See, Molecular Probes
       Product information Sheets MP 11360, MP 11350, MP 07572 and MP 11340,
       respectively and the references cited therein.

35   The numerous SYTO dyes are nucleic acid stains that passively diffuse
       through the membranes of most cells. These cell-permeant, UV- or visible light–

5   excitable dyes have been traditionally used to stain RNA and DNA in live and dead

eukaryotic cells, as well as in gram-positive and gram-negative bacteria. These dyes

share several characteristics, including permeability to virtually all cell membranes,

including mammalian cells and bacteria; high molar absorptivity, with extinction

coefficients >50,000 cm^{-1}M^{-1} at visible absorption maxima; and low intrinsic

10   fluorescence, with quantum yields typically <0.01. As noted, the dyes are available as

blue-, green-, orange- or red-fluorescent dyes. The SYTO dyes differ from each other in

one or more characteristic(s), including cell permeability, fluorescence enhancement upon

binding nucleic acids, excitation and emission spectra, and DNA/RNA selectivity and

binding affinity (*See,* Molecular Probes, 1999, Table 8.7). The SYTO dyes are

15   compatible with a variety of fluorescence-based instruments that use laser excitation or

conventional broadband illumination sources (e.g., mercury- and xenon-arc lamps).

The cyanine dyes show differences in some physical characteristics, such

as permeability and nucleic acid specificity, that allow their distribution into distinct

classes. In addition to the SYTO dyes, Hoechst 33258 and Hoechst 33342 dyes and

20   others are also useful in the context of the present invention.

The recommended dye concentration for cell staining depends on the

assay and may vary widely but is typically 0.1–50 μM for bacteria, 0.1–100 μM for yeast

and 10 nM–50 μM for other eukaryotes.

Preferred voltage sensing compositions of the invention optionally include

25   any of a variety of other membrane-permeant nucleic acid staining dyes. For example,

hexidium iodide reagents (Molecular Probes Catalogue number H-7593) are moderately

lipophilic phenanthridinium dyes that are permeant to mammalian cells and selectively

stain almost all gram-positive bacteria in the presence of gram-negative bacteria.

Generally, both the cytoplasm and nuclei of eukaryotic cells show staining with hexidium

30   iodide; however, mitochondria and nucleoli can also be stained.

Similarly, dihydroethidium is a chemically reduced ethidium derivative

that is permeant to live cells. Dihydroethidium exhibits blue fluorescence in the

cytoplasm. Many viable cells oxidize the probe to ethidium, which then fluoresces red

upon DNA intercalation.

35   LDS 751 (Molecular Probes (1999) catalogue L-7595) is a cell-permeant

nucleic acid stain. LDS 751, which has its peak excitation at ~543 nm on dsDNA, can be

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5   excited by an argon-ion laser at 488 nm and is useful in multicolor analyses due to its

long-wavelength emission maximum (~712 nm).

ACMA (9-amino-6-chloro-2-methoxyacridine, A-1324) is a DNA

intercalator that selectively binds to poly(d(A-T)). ACMA binds to membranes in the

energized state and becomes quenched if a pH gradient forms. ACMA can be used in the

10   present invention or excluded from use. For example, in one embodiment, a nucleic acid

stain other than ACMA is used for potentiometric measurements. ACMA can also be

used in potentiometric measurements.

In addition, classes of cationic membrane permeable dyes other than

nucleic acid stains can be used in the voltage sensing compositions of the invention, e.g.,

15   in conjunction with the cationic membrane permeable nucleic acid staining dyes noted

above, and/or in combination with anionic dyes in non-equilibrium measurements of

changes in TMP, or in other applications as noted herein. Such dyes include, e.g., indo-

carbocyanine dyes, thio-carbocyanine dyes, oxa-carbocyanine dyes (*see* Molecular Probes

on-line catalogue, updated as of August 10, 2,000, at section 23.3, entitled "Slow-

20   Response Dyes;" http://www.probes.com/handbook/sections/2303.html). *See also*, Sims,

et al. (1974) "Studies on the Mechanism by Which Cyanine Dyes Measure Membrane

Potential in Red Blood Cells and Phosphatidylcholine Vesicles," Biochemistry 13, 3315;

Cabrini and Verkman (1986) "Potential-Sensitive Response Mechanism of DiS-C3(5) in

Biological Membranes," Membrane Biol 92, 171; Guillet and Kimmich (1981) "DiO-C3-

25   (5) and DiS-C3-(5): Interactions with RBC, Ghosts and Phospholipid Vesicles," J

Membrane Biol 59, 1; Rottenberg and Wu (1998) "Quantitative Assay by Flow

Cytometry of the Mitochondrial Membrane Potential in Intact Cells," Biochim Biophys

Acta 1404, 393 (1998).

Other useful dyes include amino napthylethylenyl pyridinium dyes, and

30   dialkyl amino phenyl polyphenyl pyridinium dyes. The amino napthylethylenyl

pyridinium dyes include the ANEP type dyes, e.g., listed in the Molecular Probes catalog

(Di-4-ANEPPS, Di-8-ANEPPS, Di-2-ANEPEQ, Di-8-ANEPEQ and Di-12-ANEPEQ).

Dialkyl amino phenyl polyphenyl pyridinium dyes include the RH type dyes listed in the

Molecular Probes catalog (RH160, RH237, RH 421, RH 704, RH 414, and RH 461).

35   Another class of cationic probes which can be used with the cationic

membrane permeable nucleic acid staining dyes of the invention, or in combination with

15

5   anionic dyes of the invention, e.g., in non-equilibrium measurements of changes in TMP,

or in other applications as noted herein, are rhodamine probes. Rhodamine 123

(Molecular Probes 1999 catalogue number R-302) is widely used as a structural marker

for mitochondria and as an indicator of mitochondrial activity. The methyl and ethyl

esters of tetramethylrhodamine are in use as dyes for determining transmembrane

10   potential by quantitative imaging. Quantitative transmembrane potential measurements

utilizing the Nernst equation imply that the membrane distribution of the dye depends on

the transmembrane potential and that other processes, such as dye aggregation and

potential-independent interactions with intracellular components, contribute minimally.

The methyl and ethyl esters of tetramethylrhodamine, e.g., TMRM and TMRE fulfill

15   these criteria (*See*, Molecular Probes, 1999, *supra*). They are more membrane-permeant

than rhodamine 123, and their strong fluorescence means that they can be used at low

concentrations, thus avoiding aggregation. Because their fluorescence is relatively

insensitive to the environment, spatially resolved fluorescence of TMRM and TMRE

presents an unbiased profile of membrane distribution that can be directly related to

20   transmembrane potential via the Nernst equation. This is particularly useful in

embodiments where theses cationic dyes are used in conjunction with anionic dyes in

monitoring changes in TMP under non-equilibrium dye distribution conditions.

ANIONIC MEMBRANE PERMEABLE REDISTRIBUTING DYES

As noted, preferred voltage sensing compositions of the invention include

25   any of a variety of anionic membrane permeable redistributing dyes, including which are

available and can be used in the assays, compositions and devices of the invention.

Additional new anionic dyes which developed which are membrane permeable can also

be used in the methods and systems herein.

Examples of available anionic dyes include the anionic bis-isoxazolone

30   oxonols which accumulate in the cytoplasm of depolarized cells by a Nernst equilibrium–

dependent uptake from the extracellular solution. Of the oxonols studied in one reference

("Kinetics of the Potential-Sensitive Extrinsic Probe Oxonol VI in Beef Heart

Submitochondrial Particles." J.C. Smith, B. Chance. J Membrane Biol 46, 255 (1979)),

oxonol VI gave the largest spectral shifts, with an isosbestic point at 603 nm. Oxonol VI

35   responds to changes in potential more rapidly than oxonol V.

5   The three common bis-barbituric acid oxonols, often referred to as DiBAC

dyes, form a family of spectrally distinct potentiometric probes with excitation maxima at

approximately 490 nm (DiBAC$_4$(3), 530 nm (DiSBAC$_2$(3)) and 590 nm (DiBAC$_4$(5)).

DiBAC$_4$(3) has been used in many publications that cite using a "bis-oxonol" (Molecular

Probes, 1999, chapter 23). The dyes enter depolarized cells where they bind to

10   intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral

shifts. Increased depolarization results in more influx of the anionic dye and thus an

increase in fluorescence. DiBAC$_4$(3) has particularly high voltage sensitivity. The long-

wavelength DiSBAC$_2$(3) has frequently been used in combination with the UV light–

excitable Ca^{2+} indicators indo-1 or fura-2 for the simultaneous measurements of

15   transmembrane potential and Ca^{2+} concentrations (*id.* at Table 23.2).

NON-EQUILIBRIUM TMP CHANGE MEASUREMENTS: CATIONIC, ANIONIC AND NEUTRAL DYE COMPOSITIONS

One aspect of the present invention is the surprising discovery that

measurement of dye uptake prior to equilibration of the dye across a membrane results in

20   an accurate way of measuring changes in TMP. In particular, detection of both cationic

and anionic dyes at one or more time points prior to equilibration of dye across a

membrane increases the signal to noise and dynamic range for measuring changes in

TMP. Often, a single time point measurement is sufficient to provide an indication of

changes in TMP.

25   Thus, a first component comprising one or more membrane is mixed with

at least a first membrane permeable redistributing dye and one or more signal output from

the first redistributing dye measured before an equilibrium dye distribution is established,

providing a non-equilibrium dye distribution measurement. This non-equilibrium dye

distribution measurement depends on the change in transmembrane potential. Generally,

30   at least a second membrane permeable redistributing dye is added to the one or more

component and one or more signal outputs from the second membrane permeable

redistributing dye is also measured before an equilibrium dye distribution is established.

A first signal output from the cationic dye and a second signal from the anionic dye is

measured at one or more time point, thereby providing an indication of at least one

35   change in transmembrane potential.

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5   Any of the membrane permeable nucleic acid staining dyes noted above are suitable for measuring changes in TMP by the methods herein. In addition, other cationic and/or anionic membrane redistributing dyes such as cationic rhodamines and the other cationic dyes noted herein can be used in the methods of the invention, as well as $DiBAC_4(3)$ and the other dyes noted herein. In applications using both cationic and

10   anionic dyes, any of those cationic or anionic dyes noted above can be used to generate a signal which depends on TMP. It is expected that one of skill can select optimal dye combinations simply by performing any TMP assay noted herein using the various dyes which are noted. For example, the combination of SYTO®62 and $DiBAC_4(3)$ in non-equilibrium TMP assays was found to provide for an extended dynamic range for

15   monitoring changes in the TMP assay and to provide a high signal to noise ratio in the assays.

The anionic and cationic dyes can be pre-mixed and added to a membrane preparation, e.g., in a microfluidic system, or the dyes can be separately added to the membrane preparation. The decision whether to pre-mix the dyes depends on, e.g., the

20   source of the dyes, the preference of the user, the type of device in use, and the compatibility of the dyes to be mixed. In an alternate embodiment, anionic and cationic dyes are used in separate assays, on the same or separate membrane preparations, with signal measurements from anionic and cationic dyes being taken at similar or different time points and the data combined by any mathematical method to produce combinatorial

25   data sets, graphs, or the like. Thus, dyes can be combined prior to contacting membrane preparations, or can be independently contacted to the same or different membrane preparations. Data sets produced from signal measurements from distinct dyes can be analyzed independently or can be combined.

Neutral dyes can be used in the methods and as a feature of the

30   compositions of the invention. In particular, neutral dyes are useful controls in many of the methods herein. For example, a neutral dye can be used to monitor membrane permeability, including changes in permeability which are not voltage dependent. For example, the effects of temperature, pH, solvents, or the like on permeability can be monitored using a neutral dye, and the changes in permeability due to temperature effects

35   can be used in normalizing or otherwise interpreting data obtained for cationic and/or anionic dyes. Similarly, a cell or other membrane containing structure can be monitored

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5   for being intact by monitoring a signal output from the neutral dye. In general, neutral

dyes are selected such that they do not substantially interfere with other components of

the assay (cationic or anionic dyes, membranes, etc.).

In view of the use of neutral dyes in the assays as set forth herein, the

various microscale devices herein can also include a source of a neutral dye, means for

10   flowing the dye into contact with membrane preparations and the like.

A variety of neutral dyes are set forth in the references herein, including

Molecular Probes (1999). Such dyes can be polar or non-polar, including neutral red,

nonpolar pyrene probes, non-polar BODIPY probes, Nile Red, amphiphilic derivatives of

various rhodamines, fluoresceins and coumarins and many others.

15   MEMBRANE PREPARATIONS

One of skill can adapt available cells, membrane preparations and other

reagents to the present invention by providing the cells, membrane preparations and other

reagents to the microfluidic systems herein as noted. Assay conditions and buffer and

reagent parameters utilizing dyes, modulators, cells, membrane preparations and the like

20   can be selected based upon established TMP activity levels, known pairings of reagents,

concentrations and kinetic information for known TMP modulators, modified by the

addition of a selected modulator or putative modulator to the system. Initial screens of a

particular modulator or putative modulator can be conducted at a single concentration in

the system, or at multiple modulator concentrations. Typically, compounds which have

25   modulatory activity based upon an initial screen are titrated into contact with the

membrane, in increasing or decreasing amounts, to establish a dose-response curve for the

modulator.

Rather than simply using cells which naturally display TMP response to

modulators under some set of conditions, recombinant cells can also be constructed which

30   incorporate desired TMP response activities. This is advantageous because certain cells

can be easily maintained in culture using established methods.

In general, methods of making recombinant cells and expressing cellular

proteins such as membrane channel proteins and transporter receptors are well known in

the art. For an introduction to recombinant methods, see, Berger and Kimmel, Guide to

35   Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press,

Inc., San Diego, CA (Berger); Sambrook et al., Molecular Cloning - A Laboratory

5   Manual (2nd ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New
York, 1989 ("Sambrook"); and Current Protocols in Molecular Biology, F.M. Ausubel et
al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc.
and John Wiley & Sons, Inc., (supplemented through 1999) ("Ausubel").

Furthermore, the culture of cell lines and cultured primary cells from tissue
10   or blood samples is well known in the art. Freshney (Culture of Animal Cells, a Manual
of Basic Technique, third edition Wiley-Liss, New York (1994)) and the references cited
therein provides a general guide to the culture of animal cells. The culture of Mammalian
Cells is described in Freshney, id., and in Doyle and Griffiths (1997) Mammalian Cell
Culture: Essential Techniques John Wiley and Sons, NY. The culture of plant cells is
15   described in Payne et al. (1992) Plant cell and tissue culture in liquid systems John Wiley
& Sons, Inc. New York, NY. Additional information on cell culture, including
prokaryotic cell culture, is found in Ausubel, Sambrook and Berger, supra. Cell culture
media are described in Atlas and Parks (eds) The Handbook of Microbiological Media
(1993) CRC Press, Boca Raton, FL. Additional information is found in commercial
20   literature such as the Life Science Research Cell Culture catalogue (1998) from Sigma-
Aldrich, Inc (St Louis, MO) and, e.g., the Plant Culture Catalogue and supplement (1997)
also from Sigma-Aldrich, Inc (St Louis, MO). Many cells can be tested for TMP
response to a selected modulator. In the context of the invention, cells which can be
tested include those which have cell walls and/or cell membranes which are permeable to
25   the dyes being used.

Ionic channels are pores in membranes (e.g., cell membranes) which
mediate passage of many ions across the relevant membrane. Hille (1992) Ionic Channels
of Excitable Membranes, second edition, Sinauer Associates Inc. Sunderland, MA (ISBN
0-87893-323-9) (Hille) provide an introduction to ionic channels. Example channel types
30   are those which permit NA^+, K^+, Ca^{++} or other ions to pass through the membrane.
Details regarding the structure and function of these and other ion channels is found in
Hille. Membrane preparation techniques comprising ion channels are found in Hille and
the references cited therein.

Another set of proteins which can affect TMP responses are transporter
35   proteins. These proteins actively transport ions and other molecules into or out of cells.
details regarding the cloning and expression of transporters is found in Neurotransmitter

5   Transporters: Structure, Function and Regulation (1997) M.E.A Reith, ed. Human Press,

Towata NJ; Neurotransmitter Methods: Methods in Molecular Biology Volume 72 (1997)

R. Rayne, ed. Human Press, Towata, NJ; Neuropeptide Protocols: Methods in Molecular

Biology Volume 73: Irvine and Williams, eds., Human Press, Towata NJ;

Neurochemistry: A Practical Approach, 2nd edtion (1997) Turner and Bachelard, eds.,

10   Oxford Press, Oxford England; and, Neural Cell Culture: A Practical Approach (1996)

Cohen and Wilkins, eds. Oxford Press, Oxford England.

The above references also provide a number of membrane preparation

protocols for producing a variety of membrane preparations useful in the present

invention. These include cell preparations, liposome preparations and the like. A variety

15   of additional references teach a variety of such membrane preparatory techniques, e.g.,

Graham and Higgins (1997) Membrane Analysis Bios Scientific Publishers, Oxford,

England; Gould (Ed) (1994) Membrane Protein Expression Systems: A User's Guide

Portland Press, London, England; Gunstone (1996) Fatty Acid And Lipid Chemistry

Blackie Academic and Professional, London, England; Yehuda and Mostofsky (Eds.)

20   (1997) Handbook of Essential Fatty Acid Biology: Biochemistry, Physiology and

Behavioral Neurobiology Humana Press, Towata, NJ; Riafai and Warnick (1994)

Laboratory Measurement of Lipids, Lipoproteins and Apolipoproteins AACC Press,

Washington, D.C., and New (Ed) (1990) Liposomes: A Practical Approach IRL Press at

Oxford, England.

25   TMP MEASURMENTS

Potentiometric measurements are made over time to determine changes in

TMP over time, e.g., in response to TMP modulators. The measurements are made by

monitoring, e.g., changes in fluorescence over time. Several distinct emission

wavelengths can be monitored simultaneously. As discussed below, a variety of

30   microfluidic systems incorporate fluorescence detectors which can be used in the context

of the present invention. In addition, fluorescence can be monitored in standard cuvettes

and/or microtiter plates, using spectrophotometers and plate readers common in the art.

Ordinarily, *changes* in fluorescence are monitored and correlated to

changes in TMP. However, static measurements (e.g., single time point measurements)

35   can also be made and correlated to expected fluorescence measurements (e.g., compared

to collected (e.g., tabulated) fluorescence information) e.g., for calibration purposes.

5   TMP MODULATORS

A variety of ionic channel activity modulators are known. For example many neurotoxins block specific ion channels, and/or modify the kinetics of channel gating. *See*, Hille at chapter 17. For example, treatment of cells with pronase results in slowing or loss of Na^+ inactivation.

10   A variety of agents which modify gating in NA^+ and other ion channels are known and available, such as those listed in Table 1 of Hille at chapter 17. Examples include chemical agents which eliminate TMP inactivation such as pronase, trypsin, NBA, NBS, TNBS, SITS, IO_3, trinitrophenol, Glyoxal, tannic acid, Formaldehyde, glutaraldehyde, $pH_i < 6$, $pH_i, > 9$, Acridine orange, eosine Y plus

15   light and DPI-201-106. Other examples include Scorpion and coelenterate peptide α toxins slowing membrane inactivation such as those from *Leiurus quinquestriatus* (North African), *Buthus eupeus; B. tamalus* (Asian), *Androctonus australis* (North African), *Centruroides sculpturatus; C. suffusus* (North American), *Tityus serrulatus* (South American), *Anemonia sulcata* (Mediterranean), *Anthopleura*

20   *xanthogrammica* (California), and *Condylactis gigantea* (Bermuda). Other examples include Scorpion peptide β toxins shifting membrane activation such as *Centruroides sculpturatus, C. suffusus* and *Tityus serrulatus*. Other examples include lipid-soluble toxins shifting activation and slowing inactivation such as Aconitine, veratridine, and batrachotoxin. Similarly, Pyrethroids such as

25   allethrin, dieldrin, aldrin, and tetramethrin have an effect on TMP and/or alterations in TMP. Similarly, grayanotoxins, DDT and analogs affect TMP and changes in TMP. A variety of ionic conditions affect voltage dependence of gating, such as the presence of external divalent ions and pH, external and internal monovalent ions, and the presence of charged or dipolar adsorbants such as

30   lyotropic anions, salicylates and phlorizin.

Many molecules can be tested for TMP modulatory activity using the methods herein. Preferably, the molecules which are tested do not interact directly with the dyes used in the assays, as any such interaction complicates interpretation of any results which are observed. Similarly, a variety of chemical compounds can be used as

35   potential modulators in the assays of the invention, although, most often, the compounds

5   do not interact directly with the dyes used in the assays and, commonly, compounds

which can be dissolved in aqueous or organic (e.g., DMSO-based) solutions are used to

facilitate flow, e.g., where the assays are conducted in microscale systems. The assays

herein are designed to optionally screen large chemical libraries by automating the assay

steps and providing compounds from any convenient source to the assays. It will be

10   appreciated that there are many suppliers of chemical and biological compounds,

including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis,

MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

         In one preferred embodiment, high throughput screening methods involve

providing a combinatorial library containing a large number of potential TMP activity

15   modulator compounds ("potential modulator compounds"). Such "combinatorial

chemical libraries" are screened in one or more assays, as described herein, to identify

those library members (particular chemical species or subclasses) that display a desired

characteristic activity. The compounds thus identified can serve as conventional "lead

compounds" or can themselves be used as potential or actual therapeutics for treating

20   conditions amenable to treatment by modulating TMP activities. For example, a variety

of diseases are treated by administering TMP modulators, such as transport modulators,

including, e.g.,: panic, stress, obsessive compulsive disorders, depression, chronic pain

and many other physical and psychological conditions. *See*, Neurotransmitter

Transporters: Structure, Function and Regulation (1997) M.E.A Reith, ed. Human Press,

25   Towata NJ, and the references cited therein.

         A typical combinatorial chemical library is a collection of diverse

chemical compounds generated by either chemical synthesis or biological synthesis, by

combining a number of chemical "building blocks" such as reagents. For example, a

linear combinatorial chemical library such as a polypeptide library is formed by

30   combining a set of chemical building blocks (amino acids) in every possible way, or a

selected way for a given compound length (i.e., the number of amino acids in a

polypeptide compound). Millions of chemical compounds can be synthesized through

such combinatorial mixing of chemical building blocks.

         Preparation and screening of combinatorial chemical libraries is well

35   known to those of skill in the art. Such combinatorial chemical libraries include, but are

not limited to, peptide libraries (see, e.g., U.S. Patent 5,010,175, Furka, Int. J. Pept. Prot.

5   Res. 37:487-493 (1991) and Houghton et al., Nature 354:84-88 (1991)). Other

chemistries for generating chemical diversity libraries can also be used. Such chemistries

include, but are not limited to: peptoids (PCT Publication No. WO 91/19735), encoded

peptides (PCT Publication WO 93/20242), random bio-oligomers (PCT Publication No.

WO 92/00091), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as

10   hydantoins, benzodiazepines and dipeptides (Hobbs et al., Proc. Nat. Acad. Sci. USA

90:6909-6913 (1993)), vinylogous polypeptides (Hagihara et al., J. Amer. Chem. Soc.

114:6568 (1992)), nonpeptidal peptidomimetics with α-D- glucose scaffolding

(Hirschmann et al., J. Amer. Chem. Soc. 114:9217-9218 (1992)), analogous organic

syntheses of small compound libraries (Chen et al., J. Amer. Chem. Soc. 116:2661

15   (1994)), oligocarbamates (Cho et al., Science 261:1303 (1993)), and/or peptidyl

phosphonates (Campbell et al., J. Org. Chem. 59:658 (1994)), nucleic acid libraries (see,

Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology

volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al. (1989)

Molecular Cloning - A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Spring Harbor

20   Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and Current Protocols in

Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between

Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (e.g., current through

1999, e.g., at least through supplement 37) (Ausubel)), peptide nucleic acid libraries (see,

e.g., U.S. Patent 5,539,083), antibody libraries (see, e.g., Vaughn et al., Nature

25   Biotechnology, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (see,

e.g., Liang et al., Science, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small

organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, Jan 18, page 33

(1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S.

Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino

30   compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like). Diverse

shuffled libraries of nucleic acids are optionally provided, e.g., using fast forced evolution

techniques, e.g., as in 5,605,793, 5,811,238, 5,830,721, 5,834,252 and 5,837,458.

Devices for the preparation of combinatorial libraries are commercially

available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY,

35   Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus,

Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves

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5   commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru,

Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton,

PA, Martek Biosciences, Columbia, MD, etc.).

Control reactions which measure TMP or activity of a selected TMP

modulator which does not include the modulator are optional, as the assays can be

10   performed in a uniform fashion. Such optional control reactions are generally

appropriate, however, and increase the reliability of the assay(s). Accordingly, in one

embodiment, the methods of the invention include a control reaction (or reactions). For

each of the assay formats described, "no modulator" control reactions which do not

include a modulator provide a background level of transporter activity. "Control

15   modulator" reactions which have a known activity on TMP in a particular assay can also

be run.

In some assays, it is desirable to have positive controls to ensure that the

components of the assays are working properly. At least two types of positive controls

are appropriate. First, a known activator of ion channels, ionophores, or ion transporters

20   can be flowed into contact with a membrane component comprising transport activities,

and the resulting affects on TMP activity monitored. Second, a known inhibitor of ion

channels or transporters can be added, and the resulting effects in TMP activity similarly

detected. It will be appreciated that modulators can also be combined in assays with

known activators or inhibitors to find modulators which inhibit activation or repression of

25   activity by the known activator or inhibitor.

TMP ASSAYS

In accordance with the methods of the invention, the change in the level of

fluorescence of the composition is detected, where the change in fluorescence is

indicative of a change in transmembrane potential. Typically, the assay methods

30   described herein are used to detect the effect of some stimulus on the functioning of a

cellular system. Where one is seeking to determine the effect of some stimulus on a cell's

transmembrane potential, e.g., through a change in ion flux, transport, membrane

permeability, or the like, one need only expose the cell to that stimulus and examine the

cell for the presence of a previously absent fluorescent signal (or the absence of a

35   previously present fluorescent signal). Of particular interest are the effects of chemical

5   compounds, e.g., drug candidates, on cellular functioning, as determinable from TMP
       measurements.

              For example, in one assay format, a dye is contacted to a membrane
       composition. In accordance with these methods, the membrane composition is typically
       placed into a reaction vessel, such as a microfluidic channel, and the level of fluorescence

10   from the composition is measured, optionally over a period of time. This can be used to
       provide an initial or background level of fluorescence indicative of an existing
       transmembrane potential for the cell population. The particular stimulus that is to be
       tested is then inflicted upon the cell population. For example, a pharmaceutical candidate
       or test compound is added to the cell population. Following this stimulus, the

15   fluorescence level of the cells is again measured (typically over time) and compared to
       the initial fluorescent level or the fluorescence level in a control cell population (e.g.,
       which is exposed to a control TMP modulator). Any change in the level of fluorescence
       not attributable to dilution by the test compound (as determined from an appropriate
       control) is then attributable to the effect the test compound has on the cell's

20   transmembrane potential, or rate of TMP change in response to depolarization or
       hyperpolarization events.

              As described in greater detail below, typically, these types of reactions are
       carried out in an appropriate reaction receptacle that allows measurement of fluorescence,
       in situ. As such, the receptacle is typically a transparent reaction vessel, such as a test

25   tube, cuvette, a reaction well in a multiwell plate, or a transparent conduit, e.g., a
       capillary, microchannel or tube. In particularly preferred aspects, the assay methods are
       carried out in the channel or channels of a microfluidic device, as described in greater
       detail below.

              The assay methods of the present invention are particularly useful in
       performing high-throughput (greater than 1,000 compounds/day) and even ultra-high

30   performing high-throughput (greater than 1,000 compounds/day) and even ultra-high
       throughput (e.g., greater than 10,000 compounds/day) screening of chemical libraries,
       e.g., in searching for pharmaceutical leads. These experiments may be carried out in
       parallel by a providing a large number of reaction mixtures (e.g., cell suspensions as
       described herein) in separate receptacles, typically in a multiwell format, e.g., 96 well,

35   324 well or 1536 well plates. Different test compounds (library members) are added to
       separate wells, and the effect of the compound on the reaction mixture is ascertained, e.g.,

26

5   via the fluorescent signal. These parallelized assays are generally carried out using

specialized equipment to enable simultaneous processing of large numbers of samples,

i.e., fluid handling by robotic pipettor systems and fluorescent detection by multiplexed

fluorescent multi-well plate readers.

In an alternative aspect, the assays are carried out, at least in part, in a

10   serial format, where separate samples are screened one after another for an effect on a

cellular system or other membrane preparation. In order to expand throughput, these

individual serial processing units themselves may be multiplexed or parallelized. In

particularly preferred aspects, the serial assays are performed within a microfluidic device

or system. Examples of these microfluidic devices and systems are described in

15   Published International Patent Application No. WO 98/00231, which is incorporated

herein by reference in its entirety for all purposes.

ASSAY SYSTEMS

The present invention provides assay systems for carrying out the assay

methods of the present invention. Briefly and as noted above, such systems typically

20   employ a reaction or assay receptacle in which the compositions of the invention are

disposed. Additional reagents may be added, e.g., as potential or actual inhibitors or

enhancers of the reaction of interest (involving TMP measurement). Typically, the

receptacle includes at least a portion that is transparent, so that a fluorescent signal from

the dye may be detected. Of course, in the case of test tubes or wells, detection can be

25   made through an opening in the receptacle, e.g., the top opening of a well. A variety of

receptacles are useful in the present invention, including individual test tubes, cuvettes,

wells in a multiwell plate, or capillary tubes.

Microfluidic And Integrated Systems For TMP Measurements And High
Throughput Detection Of TMP Modulators
30   A variety of microscale systems which can be adapted to the present

invention by incorporating transporter components, transmitter components, modulators

and the like are available. Microfluidic devices which can be adapted to the present

invention by the addition of TMP assay components are described in various PCT

applications and issued U.S. Patents by the inventors and their coworkers, including U.S.

35   Patent Nos. 5,699,157 (J. Wallace Parce) issued 12/16/97, 5,779,868 (J. Wallace Parce et

al.) issued 07/14/98, 5,800,690 (Calvin Y.H. Chow et al.) issued 09/01/98, and 5,842,787

5   (Anne R. Kopf-Sill et al.) issued 12/01/98; and published PCT applications, such as, WO
       98/00231, WO 98/00705, WO 98/00707, WO 98/02728, WO 98/05424, WO 98/22811,
       WO 98/45481, WO 98/45929, WO 98/46438, and WO 98/49548.

               For example, pioneering technology providing cell based microscale
       assays are set forth in Parce et al. "High Throughput Screening Assay Systems in

10   Microscale Fluidic Devices" WO 98/00231 and, e.g., in PCT/US00/04522 filed
       02/22/2000, entitled MANIPULATION OF MICROPARTICLES IN MICROFLUIDIC
       SYSTEMS, by Mehta et al. Complete integrated systems with fluid handling, signal
       detection, sample storage and sample accessing are available. For example, Parce et al.
       "High Throughput Screening Assay Systems in Microscale Fluidic Devices" WO

15   98/00231 provide pioneering technology for the integration of microfluidics and sample
       selection and manipulation. Additional references which provide additional details on
       manipulating cells in microfluidic systems, e.g., in cell focusing applications, cell sorting
       applications high-throughput cell-based assays and the like, all of which can be practiced
       in the context of the present invention, include: FOCUSING OF MICROPARTICLES IN

20   MICROFLUIDIC SYSTEMS by Wada et al., Application No: PCT/US00/13294, filed
       05/11/2000, and HIGH THROUGPUT METHODS, SYSTEMS AND APPARATUS
       FOR PERFORMING CELL BASED SCREENING ASSAYS by Wada et al., application
       no: PCT/US99/13918, filed 06/21/1999.

               In general, cells, modulators, dyes, membrane components and other

25   elements can be flowed in a microscale system by electrokinetic (including either
       electroosmotic or electrophoretic) techniques, or using pressure-based flow mechanisms,
       or combinations thereof.

               Cells in particular are desirably flowed using pressure-based flow
       mechanisms. Pressure forces can be applied to microscale elements to achieve fluid

30   movement using any of a variety of techniques. Fluid flow (and flow of materials
       suspended or solubilized within the fluid, including cells or other particles) is optionally
       regulated by pressure based mechanisms such as those based upon fluid displacement,
       e.g., using a piston, pressure diaphragm, vacuum pump, probe or the like to displace
       liquid and raise or lower the pressure at a site in the microfluidic system. The pressure is

35   optionally pneumatic, e.g., a pressurized gas, or uses hydraulic forces, e.g., pressurized
       liquid, or alternatively, uses a positive displacement mechanism, i.e., a plunger fitted into

5   a material reservoir, for forcing material through a channel or other conduit, or is a combination of such forces.

In other embodiments, a vacuum source is applied to a reservoir or well at one end of a channel to draw the suspension through the channel. Pressure or vacuum sources are optionally supplied external to the device or system, e.g., external vacuum or

10   pressure pumps sealably fitted to the inlet or outlet of the channel, or they are internal to the device, e.g., microfabricated pumps integrated into the device and operably linked to the channel. Examples of microfabricated pumps have been widely described in the art. *See, e.g.*, published International Application No. WO 97/02357.

Hydrostatic, wicking and capillary forces can also be used to provide

15   pressure for fluid flow of materials such as cells. See, e.g., "METHOD AND APPARATUS FOR CONTINUOUS LIQUID FLOW IN MICROSCALE CHANNELS USING PRESSURE INJECTION, WICKING AND ELECTROKINETIC INJECTION," by Alajoki et al., Attorney Docket Number 017646-007010, USSN 09/245,627, filed February 5, 1999. In these methods, an adsorbent material or branched capillary structure

20   is placed in fluidic contact with a region where pressure is applied, thereby causing fluid to move towards the adsorbent material or branched capillary structure.

Mechanisms for reducing adsorption of materials during fluid-based flow are described in "PREVENTION OF SURFACE ADSORPTION IN MICROCHANNELS BY APPLICATION OF ELECTRIC CURRENT DURING

25   PRESSURE- INDUCED FLOW" filed 05/11/1999 by Parce et al., Attorney Docket Number 01-78-0, USSN 09/310,027. In brief, adsorbtion of cells, TMP modulators, dyes, potential modulators and other materials to channel walls or other microscale components during pressure-based flow can be reduced by applying an electric field such as an alternating current to the material during flow.

30   Mechanisms for focusing cells and other components into the center of microscale flow paths, which is useful in increasing assay throughput by regularizing flow velocity is described in "FOCUSING OF MICROPARTICLES IN MICROFLUIDIC SYSTEMS" by H. Garrett Wada et al. Application No: PCT/US00/13294, filed 05/11/2000. In brief, cells are focused into the center of a

35   channel by forcing fluid flow from opposing side channels into the main channel comprising the cells, or by other fluid manipulations. Diffusible materials such as the

29

5   transmitters of the present invention are also optionally washed from cells as described by
Wada et al. during flow of the cells, i.e., by sequentially flowing buffer into a channel in
which cells are flowed and flowing the buffer back out of the channel.

In an alternate embodiment, microfluidic systems can be incorporated into
centrifuge rotor devices, which are spun in a centrifuge. Fluids and particles travel

10   through the device due to gravitational and centripetal/centrifugal pressure forces.

One method of achieving transport or movement of dyes, TMP
modulators, and even cells (particularly dyes and modulators) through microfluidic
channels is by electrokinetic material transport. "Electrokinetic material transport
systems," as used herein, include systems that transport and direct materials within a

15   microchannel and/or chamber containing structure, through the application of electrical
fields to the materials, thereby causing material movement through and among the
channel and/or chambers, i.e., cations will move toward a negative electrode, while
anions will move toward a positive electrode. For example, movement of fluids toward
or away from a cathode or anode can cause movement of transmitters, cells, modulators,

20   etc. suspended within the fluid. Similarly, the transmitters, cells, modulators, etc. can be
charged, in which case they will move toward an oppositely charged electrode (indeed, in
this case, it is possible to achieve fluid flow in one direction while achieving particle flow
in the opposite direction). In this embodiment, the fluid can be immobile or flowing and
can comprise a matrix as in electrophoresis.

25   In general, electrokinetic material transport and direction systems also
include those systems that rely upon the electrophoretic mobility of charged species
within the electric field applied to the structure. Such systems are more particularly
referred to as electrophoretic material transport systems. For electrophoretic applications,
the walls of interior channels of the electrokinetic transport system are optionally charged

30   or uncharged. Typical electrokinetic transport systems are made of glass, charged
polymers, and uncharged polymers. The interior channels are optionally coated with a
material which alters the surface charge of the channel.

A variety of electrokinetic controllers and systems are described, e.g., in
Ramsey WO 96/04547, Parce et al. WO 98/46438 and Dubrow et al., WO 98/49548, as

35   well as a variety of other references noted herein.

5   Use of electrokinetic transport to control material movement in interconnected channel structures was described, e.g., in WO 96/04547 and US 5,858,195 to Ramsey. An exemplary controller is described in U.S. 5,800,690. Modulating voltages are concomitantly applied to the various reservoirs to affect a desired fluid flow characteristic, e.g., continuous or discontinuous (e.g., a regularly pulsed field causing the

10   sample to oscillate direction of travel) flow of labeled components toward a waste reservoir. Particularly, modulation of the voltages applied at the various reservoirs can move and direct fluid flow through the interconnected channel structure of the device.

Sources Of Assay Components And Integration With Microfluidic Formats

15   Sources of membrane containing components such as cells or cell fractions, sources of dyes and sources of potential modulators can be fluidly coupled to the microchannels noted herein in any of a variety of ways. In particular, those systems comprising sources of materials set forth in Knapp et al. "Closed Loop Biochemical Analyzers" (WO 98/45481; PCT/US98/06723) and Parce et al. "High Throughput

20   Screening Assay Systems in Microscale Fluidic Devices" WO 98/00231 and, e.g., in PCT/US00/04522 filed 02/22/2000, entitled MANIPULATION OF MICROPARTICLES IN MICROFLUIDIC SYSTEMS, by Mehta et al. are applicable.

In these systems, a "pipettor channel" (a channel in which components can be moved from a source to a microscale element such as a second channel or reservoir) is

25   temporarily or permanently coupled to a source of material. The source can be internal or external to a microfluidic device comprising the pipettor channel. Example sources include microwell plates, membranes or other solid substrates comprising lyophilized components, wells or reservoirs in the body of the microscale device itself and others.

For example, the source of a cell type, component, or modulator reagent

30   can be a microwell plate external to the body structure, having, e.g., at least one well with the selected cell type or reagent. Alternatively, a well disposed on the surface of the body structure comprising the selected cell type, component, or reagent, a reservoir disposed within the body structure comprising the selected cell type, component or reagent; a container external to the body structure comprising at least one compartment comprising

35   the selected particle type or reagent, or a solid phase structure comprising the selected cell type or reagent in lyophilized or otherwise dried form.

5   A loading channel region is optionally fluidly coupled to a pipettor channel with a port external to the body structure, e.g., as depicted in Figures 6, and 7. The loading channel can be coupled to an electropipettor channel with a port external to the body structure, a pressure-based pipettor channel with a port external to the body structure, a pipettor channel with a port internal to the body structure, an internal channel

10   within the body structure fluidly coupled to a well on the surface of the body structure, an internal channel within the body structure fluidly coupled to a well within the body structure, or the like. Example configurations are depicted in the figures herein.

As described more fully herein, the integrated microfluidic system of the invention can include a very wide variety of storage elements for storing reagents to be

15   assessed. These include well plates, matrices, membranes and the like. The reagents are stored in liquids (e.g., in a well on a microtiter plate), or in lyophilized form (e.g., dried on a membrane or in a porous matrix), and can be transported to an array component of the microfluidic device using conventional robotics, or using an electropipettor or pressure pipettor channel fluidly coupled to a reaction or reagent channel of the

20   microfluidic system.

In general, the test modulator compounds are separately introduced into the assay systems described herein, or at least introduced in relatively manageable pools of modulator materials. The relative level of a particular TMP function is then assessed in the presence of the test compound, and this relative level of function is then compared

25   to a control system, which lacks an introduced test modulator compound. Increases or decreases in relative cellular function are indicative that the test compound is an enhancer or an inhibitor of the particular cellular function, respectively.

DETECTORS AND INTEGRATED SYSTEMS

Although the devices and systems specifically illustrated herein are

30   generally described in terms of the performance of a few or one particular operation, it will be readily appreciated from this disclosure that the flexibility of these systems permits easy integration of additional operations into these devices. For example, the devices and systems described will optionally include structures, reagents and systems for performing virtually any number of operations both upstream and downstream from the

35   operations specifically described herein. Such upstream operations include sample handling and preparation operations, e.g., cell separation, extraction, purification, culture, .

5   amplification, cellular activation, labeling reactions, dilution, aliquotting, and the like.
     Similarly, downstream operations may include similar operations, including, e.g.,
     separation of sample components, labeling of components, assays and detection
     operations, electrokinetic or pressure-based injection of components into contact with
     cells or other membrane preparations, or materials released from cells or membrane
10   preparations, or the like.

                   Upstream and downstream assay and detection operations include, without
     limitation, cell fluorescence assays, cell activity assays, probe interrogation assays, e.g.,
     nucleic acid hybridization assays utilizing individual probes, free or tethered within the
     channels or chambers of the device and/or probe arrays having large numbers of different,
15   discretely positioned probes, receptor/ligand assays, immunoassays, and the like. Any of
     these elements can be fixed to array members, or fixed, e.g., to channel walls, or the like.

                   Instrumentation for high throughput optical screening of cell assays is
     available. In addition to the many microfluidic systems noted herein, other automated
     approaches can also be practiced with the dyes and methods of the invention. For
20   example, the FLIPR (Fluorescence Imaging Plate Reader) was developed to perform
     quantitative optical screening for cell based kinetic assays (Schroder and Neagle (1996)
     "FLIPR: A New Instrument for Accurate, High Throughput Optical Screening" Journal of
     Biomolecular Screening 1(2):75-80). This device can be adapted to the present
     invention, e.g., by using the dyes of the invention in the indicated methods. For example,
25   cationic DNA membrane permeable dyes can be used for potentiometric measurements.
     Similarly, by acquiring data on dye uptake prior to establishing equilibrium for both
     cationic and anionic dyes, the system can be adapted to the invention.

                   Instrumentation
                   In general in the present invention, materials such as cells and dyes are
30   optionally monitored and/or detected so that an activity such as TMP activity can be
     determined. Depending on the label signal measurements, decisions can be made
     regarding subsequent fluidic operations, e.g., whether to assay a particular modulator in
     detail to determine kinetic information.

                   The systems described herein generally include microfluidic devices, as
35   described above, in conjunction with additional instrumentation for controlling fluid
     transport, flow rate and direction within the devices, detection instrumentation for

5   detecting or sensing results of the operations performed by the system, processors, e.g.,

computers, for instructing the controlling instrumentation in accordance with

preprogrammed instructions, receiving data from the detection instrumentation, and for

analyzing, storing and interpreting the data, and providing the data and interpretations in a

readily accessible reporting format.

10   Controllers
                       A variety of controlling instrumentation is optionally utilized in

conjunction with the microfluidic devices described above, for controlling the transport

and direction of fluids and/or materials within the devices of the present invention, e.g.,

by pressure-based or electrokinetic control.

15   For example, in many cases, fluid transport and direction are controlled in

whole or in part, using pressure based flow systems that incorporate external or internal

pressure sources to drive fluid flow. Internal sources include microfabricated pumps,

e.g., diaphragm pumps, thermal pumps, Lamb wave pumps and the like that have been

described in the art. See, e.g., U.S. Patent Nos. 5,271,724, 5,277,556, and 5,375,979 and

20   Published PCT Application Nos. WO 94/05414 and WO 97/02357. As noted above, the

systems described herein can also utilize electrokinetic material direction and transport

systems.

                       Preferably, external pressure sources are used, and applied to ports at

channel termini. These applied pressures, or vacuums, generate pressure differentials

25   across the lengths of channels to drive fluid flow through them. In the interconnected

channel networks described herein, differential flow rates on volumes are optionally

accomplished by applying different pressures or vacuums at multiple ports, or preferably,

by applying a single vacuum at a common waste port and configuring the various

channels with appropriate resistance to yield desired flow rates. Example systems are

30   described in USSN 09/238,467 filed 1/28/99.

                       Typically, the controller systems are appropriately configured to receive or

interface with a microfluidic device or system element as described herein. For example,

the controller and/or detector, optionally includes a stage upon which the device of the

invention is mounted to facilitate appropriate interfacing between the controller and/or

35   detector and the device. Typically, the stage includes an appropriate mounting/alignment

structural element, such as a nesting well, alignment pins and/or holes, asymmetric edge

34

5   structures (to facilitate proper device alignment), and the like. Many such configurations are described in the references cited herein.

The controlling instrumentation discussed above is also used to provide for electrokinetic injection or withdrawal of material downstream of the region of interest to control an upstream flow rate. The same instrumentation and techniques described above
10   are also utilized to inject a fluid into a downstream port to function as a flow control element.

Detector
The devices herein optionally include signal detectors, e.g., which detect fluorescence, phosphorescence, radioactivity, pH, charge, absorbance, luminescence,
15   temperature, magnetism or the like. Fluorescent detection is especially preferred and generally used for detection of voltage sensitive compounds (however, as noted, upstream and downstream operations can be performed on cells, dyes, modulators or the like, which can involve other detection methods).

The detector(s) optionally monitors one or a plurality of signals from
20   downstream of an assay mixing point in which dye and a cell or other membrane containing component with a potential modulator are mixed. For example, the detector can monitor a plurality of optical signals which correspond to "real time" assay results.

Example detectors include photo multiplier tubes, spectrophotometers, a CCD array, a scanning detector, a microscope, a galvo-scann or the like. Cells, dyes or
25   other components which emit a detectable signal can be flowed past the detector, or, alternatively, the detector can move relative to the array to determine cell position (or, the detector can simultaneously monitor a number of spatial positions corresponding to channel regions, e.g., as in a CCD array).

The detector can include or be operably linked to a computer, e.g., which
30   has software for converting detector signal information into assay result information (e.g., kinetic data of modulator activity), or the like.

Signals are optionally calibrated, e.g., by calibrating the microfluidic system by monitoring a signal from a known source.

A microfluidic system can also employ multiple different detection
35   systems for monitoring the output of the system. Detection systems of the present invention are used to detect and monitor the materials in a particular channel region (or

5   other reaction detection region). Once detected, the flow rate and velocity of cells in the

channels is also optionally measured and controlled as described above. As described in

PCT/US98/11969, and 60/142,984, correction of kinetic information based upon flow

velocity and other factors can be used to provide accurate kinetic information in flowing

systems.

10   Examples of detection systems include optical sensors, temperature

sensors, pressure sensors, pH sensors, conductivity sensors, and the like. Each of these

types of sensors is readily incorporated into the microfluidic systems described herein. In

these systems, such detectors are placed either within or adjacent to the microfluidic

device or one or more channels, chambers or conduits of the device, such that the detector

15   is within sensory communication with the device, channel, or chamber. The phrase

"within sensory communication" of a particular region or element, as used herein,

generally refers to the placement of the detector in a position such that the detector is

capable of detecting the property of the microfluidic device, a portion of the microfluidic

device, or the contents of a portion of the microfluidic device, for which that detector was

20   intended. For example, a pH sensor placed in sensory communication with a microscale

channel is capable of determining the pH of a fluid disposed in that channel. Similarly, a

temperature sensor placed in sensory communication with the body of a microfluidic

device is capable of determining the temperature of the device itself.

              Particularly preferred detection systems include optical detection systems

25   for detecting an optical property of a material within the channels and/or chambers of the

microfluidic devices that are incorporated into the microfluidic systems described herein.

Such optical detection systems are typically placed adjacent to a microscale channel of a

microfluidic device, and are in sensory communication with the channel via an optical

detection window that is disposed across the channel or chamber of the device. Optical

30   detection systems include systems that are capable of measuring the light emitted from

material within the channel, the transmissivity or absorbance of the material, as well as

the materials spectral characteristics. In preferred aspects, the detector measures an

amount of light emitted from the material, such as a fluorescent or chemiluminescent

material. As such, the detection system will typically include collection optics for

35   gathering a light based signal transmitted through the detection window, and transmitting

that signal to an appropriate light detector. Microscope objectives of varying power, field

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5   diameter, and focal length are readily utilized as at least a portion of this optical train.
       The light detectors are optionally spectrophotometers, photodiodes, avalanche
       photodiodes, photomultiplier tubes, diode arrays, or in some cases, imaging systems, such
       as charged coupled devices (CCDs) and the like. The detection system is typically
       coupled to a computer (described in greater detail below), via an analog to digital or

10   digital to analog converter, for transmitting detected light data to the computer for
       analysis, storage and data manipulation.

                  In the case of fluorescent materials such as labeled cells, the detector
       typically includes a light source which produces light at an appropriate wavelength for
       activating the fluorescent material, as well as optics for directing the light source through

15   the detection window to the material contained in the channel or chamber. The light
       source can be any number of light sources that provides an appropriate wavelength,
       including lasers, laser diodes and LEDs. Other light sources are used in other detection
       systems. For example, broad band light sources are typically used in light
       scattering/transmissivity detection schemes, and the like. Typically, light selection

20   parameters are well known to those of skill in the art.

                  The detector can exist as a separate unit, but can also be integrated with
       the controller system, into a single instrument. Integration of these functions into a single
       unit facilitates connection of these instruments with the computer (described below), by
       permitting the use of few or a single communication port(s) for transmitting information

25   between the controller, the detector and the computer.

                  ### Computer
                  As noted above, either or both of the controller system and/or the detection
       system are coupled to an appropriately programmed processor or computer which
       functions to instruct the operation of these instruments in accordance with

30   preprogrammed or user input instructions, receive data and information from these
       instruments, and interpret, manipulate and report this information to the user. As such,
       the computer is typically appropriately coupled to one or both of these instruments (e.g.,
       including an analog to digital or digital to analog converter as needed).

                  The computer typically includes appropriate software for receiving user

35   instructions, either in the form of user input into a set parameter fields, e.g., in a GUI, or
       in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different

                                                    37

5   specific operations. The software then converts these instructions to appropriate language

for instructing the operation of the fluid direction and transport controller to carry out the

desired operation. The computer then receives the data from the one or more

sensors/detectors included within the system, and interprets the data, either provides it in

a user understood format, or uses that data to initiate further controller instructions, in

10   accordance with the programming, e.g., such as in monitoring and control of flow rates,

temperatures, applied voltages, and the like.

In the present invention, the computer typically includes software for the

monitoring of materials in the channels. Additionally, the software is optionally used to

control electrokinetic or pressure modulated injection or withdrawal of material. The

15   injection or withdrawal is used to modulate the flow rate as described above, to mix

components, and the like.

Example System

Figure 6, panels A, B and C and Figure 7 provide additional details

regarding an example integrated system of the invention. As shown, body structure **102**

20   has main channel **104** fabricated therein. Cells or other membrane containing

components are flowed, e.g., from reservoir **114**, e.g., by applying a vacuum at vacuum

source **116** (and/or at any of the reservoirs or wells noted below) through main channel

104. Cells or other membrane containing components, or dye(s), or a potential modulator

or a different material such as a buffer or label can be flowed from wells **110** or **112** and

25   into main channel **104**. Cells, or dye, or a potential modulator or any additional material

can be flowed from wells **106** or **108**, or materials can be flowed into these wells, e.g.,

when they are used as waste wells, or when they are coupled to a vacuum source. Flow

from wells **114, 112, 110, 106,** or **108** can be performed by modulating fluid pressure, or

by electrokinetic approaches as described. Instead of the arrangement of channels

30   depicted in Figures 6 and 7, an arrangement such as the 3B5 microfluidic processor, as

depicted in Figure 5 can be substituted. A variety of other appropriate microfluidic

processor configurations are set forth in the references noted herein.

With respect to Figure 5, device **501** comprises cell well **503**, side well

505 (e.g., a sample well) and side well **507** (e.g., a dye well) and waste well **509**. In brief,

35   cells are flowed from well **503**, through cell feed channel **511**, and through main channel

513 to vacuum at waste well **509**. Sample is flowed from side well **505** through sample

5   feed channel **515** and into main channel **513**. Dye is flowed from side well **507** through

dye feed channel **517** and into main channel **513**. As noted, flow is directed via

application of vacuum at waste well **509**. In this particular device, the approximate

channel dimensions are 25 μm deep and 100 μm wide. Channel **511** is approximately 5.2

mm in length. Channel **515** is approximately 13.4 mm in length. Channel **517** is

10   approximately 13.4 mm in length. Channel **513** is approximately 32.3 mm in length.

Cells, membrane preparations, dyes, potential modulators or other

materials can be flowed from the enumerated wells, or can be flowed from a source

external to body **102**. As depicted, the integrated system can include pipettor channel

120, e.g., protruding from body **102**, for accessing an outside source of reagents. For

15   example, as further depicted in Figure 7, pipettor channel **120** can access microwell plate

308 which includes cells, dyes, activity modulators, controls, or the like, in the wells of

the plate. For example, a library of potential inhibitor compounds can be stored in the

wells of plate **308** for easy access by the system. TMP change inhibitors, activators or

other reagents relevant to the assays can be flowed into channel **104** through pipettor

20   channel **120**. Detector **306** is in sensory communication with channel **104**, detecting

signals resulting, e.g., from the interaction of a dye with a cell or other membrane

preparation, as described above. Detector **306** is operably linked to Computer **304**, which

digitizes, stores and manipulates signal information detected by detector **306**.

Voltage/pressure controller **302** controls voltage, pressure, or both, e.g., at the wells of the

25   system, or at vacuum couplings fluidly coupled to channel **104** (or the other channels

noted above). Optionally, as depicted, computer **304** controls voltage/pressure controller

302. In one set of embodiments, computer **304** uses signal information to select further

reaction parameters. For example, upon detecting inhibition or activation by a potential

modulator in a well from plate **308**, the computer optionally directs withdrawal of

30   additional aliquots of the potential modulator through pipettor channel **120**, e.g., to

deliver different concentrations of the potential modulator to the assay, e.g., to determine

kinetic data (such as a dose-response curve) for the potential modulator.

ASSAY KITS

The present invention also provides kits for carrying out the assay methods

35   described herein. In particular, these kits typically include the compositions described

herein, as well as additional components to facilitate the performance of the assay

5   methods by an investigator. In particular, the kits typically comprise the voltage sensing

composition of the invention.

The kit also optionally includes a receptacle in which the assay reaction is

carried out. As noted herein, the reaction receptacle is optionally a reaction vessel, i.e., a

test tube or well in a multiwell plate, or a channel or chamber region within a microfluidic

10   device. The reaction receptacle is also typically transparent, at least in part, in order to

detect the fluorescent signals from the reaction mixture.

The elements of the kits of the present invention are typically packaged

together in a single package or set of related packages. The package optionally includes

other reagents used in the assay, e.g., buffers, standard reagents, and the like, as well as

15   written instructions for carrying out the assay in accordance with the methods described

herein. In the case of prepackaged reagents, the kits optionally include pre-measured or

pre-dosed reagents that are ready to incorporate into the assay methods without

measurement, e.g., pre-measured fluid aliquots, or pre-weighed or pre-measured solid

reagents that may be easily reconstituted by the end-user of the kit.

20   Generally, the microfluidic devices described herein are optionally

packaged to include reagents for performing the device's preferred function. For

example, the kits can include any of microfluidic devices described along with assay

components, reagents, sample materials, control materials, or the like. Such kits also

typically include appropriate instructions for using the devices and reagents, and in cases

25   where reagents are not predisposed in the devices themselves, with appropriate

instructions for introducing the reagents into the channels and/or chambers of the device.

In this latter case, these kits optionally include special ancillary devices for introducing

materials into the microfluidic systems, e.g., appropriately configured syringes/pumps, or

the like (in one preferred embodiment, the device itself comprises a pipettor element,

30   such as an electropipettor for introducing material into channels and chambers within the

device). In the former case, such kits typically include a microfluidic device with

necessary reagents predisposed in the channels/chambers of the device. Generally, such

reagents are provided in a stabilized form, so as to prevent degradation or other loss

during prolonged storage, e.g., from leakage. A number of stabilizing processes are

35   widely used for reagents that are to be stored, such as the inclusion of chemical stabilizers

(i.e., enzymatic inhibitors, microcides/bacteriostats, anticoagulants), the physical

40

5   stabilization of the material, e.g., through immobilization on a solid support, entrapment

in a matrix (i.e., a gel), lyophilization, or the like.

Kits also optionally include packaging materials or containers for holding

microfluidic device, system or reagent elements, instructional materials for practicing any

of the methods noted herein, or the like.

10   EXAMPLE: DEVELOPMENT OF MICROFLUIDIC PROCESSOR-BASED
TRANSMEMBRANE POTENTIAL ASSAYS

Measurement of transmembrane potential is useful for a broad class of cell

based microfluidic assays. A widely used type of transmembrane potential assay uses

voltage-sensitive dyes which generate a fluorescent signal due to voltage-dependent, dye

15   redistribution between the inside and outside of cells. The feasibility of using such

Nernstian dyes to measure transmembrane potential in microfluidic processors was

investigated.

Typically, the use of Nernstian, voltage-sensitive dyes such as $DiBAC_4(3)$

involves the measurement of the equilibrium distribution of dye as a function of

20   transmembrane potential. Alternatively, it was found that the addition of $DiBAC_4(3)$ to

cell suspensions resulted in a voltage-dependent time course of dye uptake (Figure 1).

As shown in Figure 1, the time course of dye uptake depends on

transmembrane potential. THP-1 cells (3×10^5 cells/ml) were suspended in either high

sodium, Hepes-Hanks balanced salt solution (143 mM Na^+, 2 mM K^+) (line A) or high

25   potassium, Hepes-Hanks balanced salt solution (15 mM Na^+, 130 mM K^+) containing

15% Optiprep and 200 nM $DiBAC_4(3)$ (line B). The cells in high sodium and high

potassium buffers are expected to have transmembrane potentials of about -40 mV and 0

mV respectively. The change in $DiBAC_4(3)$ fluorescence (475 nm excitation, 520 nm

emission) after suspension of the cells was measured spectrophotometrically at 30 °C.

30   Surprisingly, it was found that the DNA stain SYTO62, a cyclic-

substituted unsymmetrical cyanine dye (see, e.g., US patent 5,436,134), acts as a voltage

sensitive dye.

In particular, as shown in Figure 2, the time course of SYTO 62 uptake

depends on transmembrane potential. THP-1 cells were suspended in high sodium (line

35   A) or high potassium (line B) buffers containing 1µM SYTO 62 as for the experiments

depicted in Figure 1. The change in SYTO62 fluorescence (475 nm excitation, 670 nm

5   emission) after cell suspension was measured spectrophotometrically at 30 °C.

Membrane permeable, cationic, ribonucleic acid stains such as SYTO62 thus represent a

new class of Nernstian voltage dyes.

Changes in the cell transmembrane potential were detected in microfluidic

processor by the mixing of cells and the dyes $DiBAC_4(3)$ and SYTO62. As shown in

10   Figure 3, transmembrane potential assays can be run in a microfluidic processor. Dye

uptake by THP-1 cells was measured on a Caliper® 3B5 microfluidic processor (Figure

5). One of the long channels of the processor contained THP-1 cells (3 x 10^6 cells/ml)

suspended in high sodium buffer as above, while the other long channel contained 400

nM $DiBAC_4(3)$ and 10 µM SYTO62 in high sodium buffer. Transmembrane potential

15   was set to resting potential, hyperpolarization or depolarization by placing either high

sodium, 50 µM UTP in high sodium or high potassium buffer in the short arm. Resting,

hyperpolarized and depolarized potentials are estimated to be approximately -40, -100

and 0 mV respectively. The microfluidic processor was maintained at room temperature

(~ 23 °C). $DiBAC_4(3)$ and SYTO62 fluorescence was read after the cells had traveled

20   approximately 90 seconds from the junction ($\Delta P \sim 1'' H_2O$). Panel A depicts the average

fluorescence ratio ($DiBAC_4(3)/SYTO62$) for runs of ~ 80 cells each. Error bars are

standard error values. As shown in panel B, Histograms show the expected voltage

dependent changes in the fluorescence intensities of individual cells as a function of

transmembrane potential. Line A shows resting cells in high sodium buffer, line B shows

25   depolarized cells in UTP/high potassium buffer and line C shows hyperpolarized cells in

UTP/high sodium buffer.

The utility of the microfluidic processors-based assay was demonstrated

by generating a Palytoxin dose-response curve as depicted in Figure 4. Dye uptake by

K562 cells (2 x 10^6 cells/ml) was measured on a 3B5 microfluidic processor as in Figure

30   3, except that readings were made ~ 70 seconds after the junction. About 100 cells per

condition were used to calculate average values and standard errors. Solutions of varying

Palytoxin concentrations were placed in the short arm to yield the indicated Palytoxin

concentrations after mixing with cells. Palytoxin opens a large Na^+ current leading to a

significant membrane depolarization.

35   The ability to measure transmembrane potential in microfluidic processors

was demonstrated. By detecting the kinetics of dye uptake rather than the equilibrium

5   dye distribution, the assay could be run quickly in microfluidic processors with high

sensitivity. Membrane permeable, cationic ribonucleic acid stains such as SYTO62 were

found to be a new class of voltage sensitive dyes. The simultaneous use of anionic and

cationic dyes allows ratiometric measurement of transmembrane potential leading to

higher sensitivity and increased dynamic range.

10   EXAMPLE: ASSAYS

The following Example illustrates the invention, but is not intended to

limit the invention in any way. One of skill will be aware of a variety of substitutions that

can be made in the following while achieving substantially similar results.

The microfluidic technology used in the LabChip® system for this example

15   utilizes microchannels etched into quartz chips coupled with a sampling micropipette that

can access samples from microtitre plates. The LabChip® system accesses liquid samples

sequentially from a microplate, mixes the samples with cells flowing through a

microchannel and reads out cellular responses using fluorescence detection. The system

was applied to the measurement of changes in transmembrane potential. Membrane

20   potential assays were demonstrated using membrane potential sensitive dyes to measure

the hyperpolarization in THP-1 cells caused by the opening of calcium-sensitive

potassium channels after addition of UTP and the depolarization caused by blocking

potassium channels by the addition of quinidine sulfate.

Lab-on-a-Chip System

25   As show in figure 8, panel A and panel B, the lab-on-a-chip system which

was used consisted of quartz chip **801** containing microchannels **803** and **805** connected

to Sipper™ capillary **807**, robot **809** to move microplate **811**, light source **813**, optics **815**

and fluorescence reader **817**, vacuum pump **816**, and a computer to control system

functions and record data. Light from light source **813** (e.g., mercury arc lamp (470 nm)

30   or an argon ion laser) was focused onto microchip **801** with through optics **815**

comprising an objective (10x, 0.3 NA air). Fluorescence was collected with the

objective, split into two channels (525 nm and 700 nm) and detected with fluorescence

reader **817** which comprises photodiodes. Fluorescence readings were acquired at 20-90

Hz. The three axis robot **809** was used to position microplate **811** so that chip **801** could

35   sample microplate **811**, whether in a 96 or 384 well configuration.

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5   A schematic of chip **801** with 90x20 μm channels is shown in Figure 8,

panel B. Flowing cells from cell well **819** were mixed with samples introduced through

the Sipper™ capillary **807** (20 μm ID). The flow rate in the detection channel was varied

from 2 to 10 nl/s by varying the vacuum applied to waste well **821**. Agonists or dyes

were added from side well **823**. The incubation time in detection channel **803** was varied

10   between 10 and 75 seconds by varying the applied pressure and the location of detector

elements (e.g., optics **815**) relative to chip **801**.

Membrane Potential Assay

As shown in figure 9, The uptake of permeant fluorophores was a sensitive

indicator of membrane potential.

15   As shown in panel A, the potential sensitive fluorescence signal was

derived not from the slow re-equilibration of dyes that were pre-loaded into cells but

rather from the rate of uptake of charged, membrane-permeable dyes. Using both an

anionic dye, $DiBAC_4(3)$, and a cationic dye, Syto 62, yielded a ratiometric measurement

with increased sensitivity. Cells were mixed on chip with test samples and dyes stored on

20   the chip. After a short incubation in the detection channel, the fluorescence of individual

cells was detected.

As shown in panel B, peaks in green (DiBAC) and red (Syto 62)

fluorescence corresponded to THP-1 cells mixed with either buffer, UTP or quinidine

sulfate flowing past the detector following a 75 second dye incubation.

25   As shown in panels C and D, the rate of uptake of $DiBAC_4(3)$ (C) and

Syto 62 (D) was determined from the average fluorescence peak height as a function of

the incubation time for resting cells (O), cells hyperpolarized with UTP (■) and cells

depolarized with quinidine sulfate (▲). Compared to resting cells, the rate of increase of

$DiBAC_4(3)$ peak heights was 48 ± 11% (mean ± se, n=5) higher for depolarized cells and

30   43 ± 5% lower for hyperpolarized cells. Similarly, the rate of increase of the Syto 62

peaks was 7 ± 2% lower for depolarized cells and 37 ± 3% higher for hyperpolarized

cells.

Depolarization and Hyperpolarization

As shown in Figure 10, depolarization and hyperpolarization were

35   measured with the highly sensitive chip based assay.

5   As shown in panel A, a calibration curve relating the ratio of

$DiBAC_4(3)$/Syto 62 fluorescence (n=20 to 130 cells) to membrane potential was

constructed by increasing the K+ conductance of the THP-1 cells and varying the

extracellular K+ concentration. The exponential curve fit indicated a doubling of the ratio

for every 33 mV change in membrane potential. The average ratio for resting THP-1

10   cells in Hank's balanced salt solution was 0.13 (n=15) yielding a resting membrane

potential of –51 mV.

           As shown in panel B, varying concentrations of UTP were sipped to

hyperpolarize THP-1 cells. The dose dependent response of the $DiBAC_4(3)$/Syto 62 ratio

(average ± SD, n=6) to UTP is shown with an EC_{50} of 0.1μM. As shown in panel C, the

15   change in the average $DiBAC_4(3)$/Syto 62 ratio (average ± SD, n=3) due to quinidine

sulfate dose dependent depolarization is shown. The average coefficient of variance was

~10% for replicate measurements of 30 to 80 cells each. From the calibration curve, this

corresponds to a 5 mV error in the estimation of the membrane potential.

Use of Primary Cells

20   As shown in Figure 11, low cell consumption and high data quality

allowed the use of primary cells. Resting or mitogen activated peripheral blood T

lymphocytes (90% CD3+) were flowed through the chip and mixed with ion channel

modulators accessed through the Sipper™ capillary. Average $DiBAC_4(3)$/Syto 62 ratios

± SE (n=2-6) are shown. Resting cells show a significant increase in $DiBAC_4(3)$/Syto 62

25   ratio (*, p< 0.05) corresponding to a depolarization in the presence of 36 μM margatoxin

(BK_{Ca} blocker), but not 30 μM clotrimazole (IK_{Ca} blocker), 200 nM apamin (SK_{Ca}

blocker) or 15 μM ionomycin (IK_{Ca} activator). In contrast, activated cells in the presence

of ionomycin had a lower ratio compared to resting T lymphocytes (*, p< 0.05)

corresponding to hyperpolarization. The ratio increases significantly in the presence of

30   30 μM clotrimazole (**, p< 0.05).

           Thus, cell based assays of calcium flux and membrane potential were run

in the lab-on-a-chip system. A novel approach for measurement of membrane potential

was used to detect hyperpolarization and depolarization with high sensitivity. The

microfluidic format reduced reagent, sample and cell consumption. As few as 50-200

35   cells and as little as 10 nl of sample were used per test. The ability to use primary cells

5   enabled by the low cell consumption was demonstrated by assaying ion channel activity in human lymphocytes.

Figure 12 shows a calibration curve relating the ratio of DiBAC4(3)/Syto 62 fluorescence to membrane potential which was constructed for RBL-2H3 cells. RBL-2H3 cells (ATCC CRL-2256) were grown in minimum essential Eagle medium with 2

10   mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 15% heat-inactivated fetal bovine serum and 100 u/ml penicillin, 100 µg/ml streptomycin at 37 µC in 5% CO2. Cells were suspended by trypsinization and placed on chip in HBSS buffer containing 18% Optiprep. Membrane potential was modulated by taking advantage of the large potassium

15   conductance and varying the extracellular concentration of KCl from 2 to 66 mM. Membrane potentials were calculated using the Goldman-Hodgkin-Katz equation. Figure 12 shows the calibration curve. The data was fit by an exponential curve (ratio= 1.2xexp[0.019xpsi(mV)]) indicating a doubling of the ratio for every 30 mV change in membrane potential. The average ratio for resting RBL-2H3 cells in Hank's balanced salt

20   solution, was 0.26 corresponding to a resting membrane potential of –80 mV.

The discussion above is generally applicable to the aspects and embodiments of the invention described in the claims.

Moreover, modifications can be made to the method and apparatus described herein without departing from the spirit and scope of the invention as claimed,

25   and the invention can be put to a number of different uses including the following:

The use of a microfluidic system for performing the TMP assays set forth herein.

The use of a microfluidic system as described herein, wherein a biochemical system flows through one of said channels substantially continuously,

30   providing for, e.g., sequential testing of a plurality of TMP modulatory compounds.

The use of pressure-based or electrokinetic injection in a microfluidic device as described herein to modulate or achieve flow of cells, membrane preparations, dyes, or other assay components in channels of a microscale device.

The optional use of a combination of adsorbent materials, electrokinetic

35   injection and pressure based flow elements in a microfluidic device as described herein to modulate or achieve flow of materials e.g., in the channels of the device.

5   An assay utilizing a use of any one of the microfluidic systems or
substrates described herein.

While the foregoing invention has been described in some detail for
purposes of clarity and understanding, it will be clear to one skilled in the art from a
reading of this disclosure that various changes in form and detail can be made without

10   departing from the true scope of the invention. For example, all the techniques and
apparatus described above may be used in various combinations. *All publications, patent
applications, patents and other documents cited in this application are incorporated by
reference in their entirety for all purposes to the same extent as if each individual
publication or patent document were individually so denoted.*

5   <u>WHAT IS CLAIMED IS:</u>

        1.   A composition, comprising:

        a first component comprising a membrane;

        a cationic membrane permeable dye; and,

        an anionic membrane permeable redistributing dye.

10   2.   The composition of claim 1, the cationic membrane permeable dye
comprising a cationic nucleic acid staining dye.

        3.   The composition of claim 1, wherein the first component comprises
one or more of: a cell, a mitochondria, a chloroplast, a cell vesicle, and an artificial
membrane.

15   4.   The composition of claim 3, wherein the cell comprises one or more
of: an intact cell, an animal cell, a plant cell, a fungal cell, a bacterial cell, a mammalian
cell, a primate cell, a rodent cell, a canine cell, a feline cell, and a livestock cell, a
cultured cell, a THP-1 cell, a COS cell, a CHO cell, a HEK cell, a HeLA cell, an NIH 3T3
cell, a primary cell, an endoderm cell, an ectoderm cell, a mesoderm cell, a cell derived
20 from a differentiated tissue, a cell derived from an undifferentiated tissue, a blood cell, a
peripheral blood cell, a nerve cell, a muscle cell, a skin cell and a bone cell.

        5.   The composition of claim 1, wherein the cationic membrane
permeable dye comprises one or more of: a Blue-fluorescent SYTO dye, a Green-
fluorescent SYTO Dye, an Orange-fluorescent SYTO dye, a Red-fluorescent SYTO dye,
25 SYTO 62, Pur-1, thiazol, aryl, 2DS-7J1, Hoechst 33258, Hoechst 33342 and hexidium
iodide; or wherein the anionic membrane permeable redistributing dye comprises one or
more of: an anionic bis-isoxazolone oxonol dye, a bis-oxonol dye, Oxonol V, Oxonol VI,
$DiBAC_4(3)$ $DiBAC_4(5)$, and $DiBAC_2(3)$.

        6.   A container or microfluidic processor comprising the composition of
30 claim 1.

        7.   A method of generating a signal output which is sensitive to
membrane potential, the method comprising:

                                              48

5   providing a first component comprising one or more membranes;

adding a cationic membrane permeable nucleic acid staining dye to the first

component; and,

monitoring a first signal output from the cationic membrane permeable nucleic

acid staining dye, wherein the first signal output is correlated with the transmembrane

10   potential across the one or more membranes.

8.   The method of claim 7, the method comprising monitoring a change

in transmembrane potential, the monitoring the first signal output comprising monitoring

a change in the first signal output over time and the correlating the first signal output with

the transmembrane potential across the one or more membrane comprising determining

15   the rate of change of the first signal over time.

9.   The method of claim 7, the providing step comprising flowing the

first component through a first microfluidic channel, which microfluidic channel

intersects a second microfluidic channel, wherein the cationic membrane permeable

nucleic acid staining dye is flowed through the first or second microchannel and into

20   contact with the at least one membrane.

10.   The method of claim 9, the method comprising flowing an anionic

membrane permeable redistributing dye or a neutral dye through the first or second

channel and into contact with the at least one membrane.

11.   The method of claim 7, further comprising exposing the one or more

25   membranes to one or more transmembrane potential modulatory compositions and

monitoring an effect of the one or more transmembrane potential modulatory

compositions on the first signal output, thereby monitoring an effect of the one or more

transmembrane potential modulatory compositions on the transmembrane potential.

12.   The method of claim 11, the one or more transmembrane potential

30   modulatory compositions comprising one or more of: a hyperpolarization buffer, a

depolarization buffer, and a compound which alters transport of an ion across the cell

membrane.

5   13.   A method of producing a signal which is dependent on transmembrane potential, the method comprising:

flowing a first mixture comprising one or more membranes and one or more voltage sensitive dyes through a first channel region; and,

monitoring at least a first signal output from at least one of the voltage sensitive

10   dyes, thereby producing a signal which is dependent on the transmembrane potential across the one or more membranes.

14.   The method of claim 13, wherein the voltage sensitive dyes comprise one or more membrane permeable redistributing dyes, which one or more membrane permeable dyes comprise one or more ionic dye and wherein the one or more membrane

15   permeable dyes are flowed from a source to the first channel region and into contact with the one or more membranes and wherein flow of the membrane permeable labels across the membrane is detected by monitoring the one or more signal outputs from the membrane permeable labels before an equilibrium distribution is reached.

15.   The method of claim 13 or 14, wherein the first mixture comprises

20   one or more of: a cationic dye, a cationic membrane permeable nucleic acid staining dye, an anionic dye and a neutral dye.

16.   The method of claim 15, wherein the one or more voltage sensitive dyes comprises an anionic dye, a cationic dye, or a cationic membrane permeable nucleic acid staining dye and one or more of: an anionic dye, Oxonol V, Oxonol VI, DiBAC$_4$(3),

25   DiBAC$_4$(5), DiBAC$_2$(3), WW781, RGA-30, a cationic dye, an indo-carbocyanine dye with a short alkyl tail, a thio-carbocyanine dye with a short alkyl tail, an oxa-carbocyanine dye with a short alkyl tail, an amino napthyletheny pyridinium dye, a dialkyl amino phenylpolyenyl pyridinium dye, a cationic membrane permeable nucleic acid staining dye, a SYTO dye, SYTO 62 and a neutral dye.

30   17.   The method of claim 13, wherein the first mixture is provided to the first channel by flowing a first component comprising one or more membrane from a source to a first channel region and flowing a labeling composition comprising the one or more voltage sensitive dyes into contact with the membrane.

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5   **18.**   The method of claim 13, further comprising: hyperpolarizing or
depolarizing the membrane, or changing a permeability property of the membrane, and
monitoring flow of the at least one voltage sensitive dye across the membrane by
monitoring the first signal output, thereby measuring changes in the transmembrane
potential.

10   **19.**   The method of claim 13, further comprising flowing at least a second
mixture comprising one or more second voltage sensitive dyes into contact with the
membrane and monitoring flow of the one or more second voltage sensitive dyes across
the membrane by monitoring at least a second signal output from the second voltage
sensitive dyes.

15   **20.**   The method of claim 7, 13 or 19, comprising monitoring the first or
second signal outputs over a selected period of time (t), which period is less than about
100 seconds.

           21.   The method of claim 20, wherein t is between about 0.1 and about 80
seconds.

20   **22.**   The method of claim 7, 13 or 19, wherein the first or second signal
output is monitored at one or more time points, which one or more time points are before
equilibration of the first voltage sensitive dyes, the at least second voltage sensitive dyes
or the cationic membrane permeable nucleic acid staining dye across the one or more
membranes.

25   **23.**   The method of claim 13, wherein the voltage sensitive dye is a
cationic membrane permeable nucleic acid staining dye.

           24.   The method of claim 7, 13 or 23, wherein a rate of dye translocation
across the membrane depends on the transmembrane potential across the membrane.

           25.   The method of claim 7 or 23, wherein the cationic membrane
30   permeable nucleic acid staining dye is a cyanine dye, or a cyclic-substituted
unsymmetrical cyanine dye.

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5   **26.** The method of claim 7 or 23, wherein the cationic membrane permeable nucleic acid staining dye is a dye selected from: a Blue-fluorescent SYTO dye, a Green-fluorescent SYTO Dye, an Orange-fluorescent SYTO dye, a Red-fluorescent SYTO dye, Pur-1, thiazol, aryl, 2DS-7J1, Hoechst 33258, Hoechst 33342 and hexidium iodide.

10   **27.** The method of claim 7 or 23, wherein the dye is the Red-fluorescent dye SYTO 62.

28. The method of claim 7 or 23, the method comprising adding an anionic membrane permeable redistributing dye to the first component or to the first mixture and measuring a second signal output from the anionic membrane permeable

15   redistributing dye, thereby providing a further indication of changes in the transmembrane potential.

29. The method of claim 28, further comprising determining a ratio of the first and second signal.

30. The method of claim 28, wherein the anionic membrane permeable

20   redistributing dye comprises one or more of: an anionic bis-isoxazolone oxonol dye, a bis-oxonol dye, Oxonol V, Oxonol VI, $DiBAC_4(3)$, $DiBAC_4(5)$, and $DiBAC_2(3)$.

31. The method of claim 28, wherein the cationic membrane permeable nucleic acid staining dye is SYTO 62, at a concentration of between about 0.01 and about 50 μM and the anionic dye is $DiBAC_4(3)$, at a concentration of between about 0.01 and

25   about 50μM.

32. The method of claim 7 or 13, wherein the first membrane is a component of an intact cell, which cell is suspended in a fluid comprising one or more ion selected from: Na^+, K^+, Cl^-, H^+, Ca^{2+}, and HCO_3^-.

33. The method of claim 7 or 13, wherein the one or more membrane is a

30   cell membrane.

34. The method of claim 33, wherein the cell membrane is present in an intact or live cell, or, wherein the cell is selected from: an animal cell, a plant cell, a

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5   fungal cell, a bacterial cell, a mammalian cell, a primate cell, a rodent cell, a canine cell, a

feline cell, a livestock cell, a cultured cell, a THP-1 cell, a COS cell, a CHO cell, a HEK

cell, a HeLA cell, an NIH 3T3 cell, a primary cell, an endoderm cell, an ectoderm cell, a

mesoderm cell, a primary cell derived from differentiated tissue, a primary cell derived

from undifferentiated tissue, a primary cell derived from blood, a primary cell derived

10   from peripheral blood, a primary cell derived from nerve, a primary cell derived from

muscle, a primary cell derived from skin and a primary cell derived from bone.

35. The method of claim 34, wherein the intact or live cell has a

transmembrane potential of about –100mV to about 10mV.

36. The method of claim 7 or 13, wherein the first signal is detected

15   optically.

37. The method of claim 7 or 13, wherein the first signal is detected at

between about 20°C and 40°C.

38. The method of claim 7 or 13, wherein monitoring the first signal

output comprises detecting one or more fluorescent emission produced by the cationic

20   membrane permeable nucleic acid staining dye or the membrane permeable label.

39. The method of claim 7 or 13, further comprising contacting the first

component with a transmembrane potential modulator and monitoring an effect of the

transmembrane potential modulator by monitoring the first signal output.

40. The method of claim 39, wherein the transmembrane potential

25   modulator is a control modulator or a test modulator.

41. The method of claim 40, wherein the control modulator is selected

from: a molecule, a neurotoxin, a set of neurotoxins, a neurotransmitter, a set of

neurotransmitters, a protein, a set of proteins, a peptide, a set of peptides, a lipid, a set of

lipids, a carbohydrate, a set of carbohydrates, an organic molecule, a set of organic

30   molecules, a drug, a set of drugs, a receptor ligand, a set of receptor ligands, an antibody,

a set of antibodies, a cytokine, a set of cytokines, a chemokine, a set of chemokines, a

5   hormone, a set of hormones, a cell, a set of cells, a protein attached to a cell, and a protein
attached to a set of cells.

42. A method of generating an optical signal which is sensitive to
transmembrane potential, the method comprising:
        providing a first component comprising one or more membrane;
10   adding a cationic membrane permeable redistributing dye to the first component;
        adding an anionic membrane permeable redistributing dye to the first component;
and,
        measuring a first signal output from the cationic dye and a second signal output
from the anionic dye, wherein one or more of the first and second signal outputs
15   comprises an optical signal output, thereby generating the optical signal which is sensitive
to the transmembrane potential.

43. The method of claim 42, wherein the cationic dye is a membrane
permeable nucleic acid staining dye or a cationic rhodamine, an indo-carbocyanine dye, a
thio-carbocyanine dye, an oxa-carbocyanine dye, an amino napthylethylenyl pyridinium
20   dye, a dialkyl amino phenyl polyphenyl pyridinium dye, or wherein the anionic
membrane permeable redistributing dye comprises one or more of : Oxonol V, Oxonol
VI, and $DiBAC_4(3)$ $DiBAC_4(5)$, $DiBAC_2(3)$.

44. The method of claim 42, further comprising adding a neutral dye to
the first component.

25   45. The method of claim 42, further comprising adding a neutral dye to
the first component, wherein the neutral dye produces a control signal output which is
dependent on one or more of: temperature, incubation time and overall membrane
permeability.

46. A method of generating an optical signal which is dependent on
30   transmembrane potential, the method comprising:
        providing a first component comprising one or more membrane;
        adding at least a first membrane permeable redistributing dye to the first
component, wherein the first membrane permeable redistributing dye comprises an ion;

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5   measuring one or more signal output from the first redistributing dye before an

equilibrium dye distribution is established, which one or more signal output comprises at

least one optical signal output, thereby providing the optical signal which is dependent on

transmembrane potential.

47. The method of claim 46, further comprising correlating the one or

10 more signal output to a change in transmembrane potential.

48. The method of claim 46, comprising adding at least a second

membrane permeable redistributing dye to the one or more component and measuring one

or more signal outputs from the second membrane permeable redistributing dye before an

equilibrium dye distribution is established.

15   49. The method of claim 48, wherein the first and second dyes are added

to the first component at approximately the same time and the signal outputs from the

first and second membrane permeable redistributing dyes are measured at approximately

the same time.

50. The method of claim 48, wherein the first and second redistributing

20 dyes comprise an anionic dye and a cationic dye.

51. The method of claim 48 or 50, wherein the first and second

redistributing dyes comprise one or more of: an anionic dye, a cationic dye, a cationic

membrane permeable nucleic acid staining dye, and a neutral dye.

52. The method of claim 48, further comprising adding at least a third

25 membrane permeable redistributing dye to the one or more component and measuring one

or more signal outputs from the third membrane permeable redistributing dye before an

equilibrium dye distribution is established.

53. The method of claim 52, wherein the first and second redistributing

dyes comprise one or more of: an anionic dye and a cationic dye and wherein the third

30 membrane permeable redistributing dye comprises a neutral dye.

55

5   **54.** The method of claim 53, wherein the signal output for the neutral dye is correlated to a temperature-dependent change in membrane permeability or a time-dependent change in membrane permeability.

55. The method of claim 51, wherein the cationic dye is a nucleic acid staining dye.

10   **56.** The method of claim 51, wherein the cationic dye comprises a nucleic acid staining dye selected from: a Blue-fluorescent SYTO dye, a Green-fluorescent SYTO Dye, an Orange-fluorescent SYTO dye, a Red-fluorescent SYTO dye, Pur-1, thiazol, aryl, 2DS-7J1, Hoechst 33258, Hoechst 33342 and hexidium iodide or wherein the anionic redistributing dye comprises one or more of Oxonol V, Oxonol VI,

15   and DiBAC$_4$(3) DiBAC$_4$(5), DiBAC$_2$(3).

57. The method of claim 42, wherein the anionic redistributing dye comprises Syto 62 and the anionic dye comprises DiBAC$_4$(3).

58. A microfluidic device for monitoring transmembrane potential, the

20   microfluidic device comprising:

a body structure having at least one microscale cavity disposed therein;

a target source of a first composition comprising at least one membrane, which target source is fluidly coupled to the at least one microscale cavity; and,

a source of one or more voltage sensitive dyes which source is fluidly coupled to

25   the at least one microscale cavity, wherein, during operation of the device, the first composition is contacted to the one or more voltage sensitive dyes in the at least one microscale channel.

59. The device of claim 58, wherein the one or more voltage sensitive dyes comprise one or more of: a cationic membrane permeable staining dye source

30   comprising one or more cationic membrane permeable dyes, which cationic membrane permeable dye source is fluidly coupled to the at least one microscale cavity, and an anionic membrane permeable redistributing dye source comprising one or more anionic

5   redistributing dye, which anionic redistributing dye source is fluidly coupled to the at

least one microscale cavity.

60.   The device of claim 58, wherein the cationic dye source is a nucleic

acid staining dye.

61.   The device of claim 58, wherein the device comprises both the

10   cationic membrane permeable staining dye source and the anionic membrane permeable

redistributing dye source, and wherein, during operation of the device, the first

composition is contacted, in the presence of the cationic membrane permeable dye and

the anionic membrane permeable redistributing dye, to at least one transmembrane

potential modulatory composition.

15   62.   The device of claim 58, the device further comprising a source of at

least one potential transmembrane potential modulatory composition, which source is

fluidly coupled to the at least one microscale cavity.

63.   The device of claim 58, further comprising a source of at least one

transmembrane potential modulatory composition, wherein, during operation of the

20   device, at least one transmembrane potential modulatory composition is contacted to one

or more of: the first composition, the cationic membrane permeable dye, or the anionic

membrane permeable redistributing dye.

64.   The device of claim 58, the device further comprising a plurality of

sources of at least one potential membrane modulatory composition.

25   65.   The device of claim 64, wherein the plurality of sources comprise

one or more microtiter trays, each of the one or more trays comprising at least one

potential membrane modulatory composition.

66.   The device of claim 65, wherein the microtiter trays are movably

mounted proximal to the body structure, wherein the body structure comprises one or

30   more pipettor channels which are structurally configured to access the trays, which one or

more pipettor channels are fluidly coupled to the at least one microscale cavity.

5   67.   The device of claim 62, wherein the at least one potential membrane
modulatory composition comprises one or more of: a membrane hyperpolarization buffer,
a membrane depolarization buffer, a compound which alters membrane permeability and
a compound which alters transport of an ion across the cell membrane.

           68.   The device of claim 58, further comprising a signal detector located
10   proximal to or within the microscale cavity, which signal detector detects the a signal.

           69.   The device of claim 68, wherein the detector detects the detectable
signal for a selected length of time (t), or a selected time point (t_p).

           70.   The device of claim 58, wherein the at least one microscale cavity is
a first microscale channel, and wherein, during operation of the device:
15   the first composition comprising the at least one membrane is flowed from the
target source into the microchannel;
            the cationic membrane permeable dye or the an anionic membrane permeable
redistributing dye is flowed into contact with the first composition; and,
            the detectable signal is monitored at one or more selected time points after contact
20   of the first composition with the cationic membrane permeable dye or the an anionic
membrane permeable redistributing dye.

           71.   The device of claim 70, wherein at least one potential membrane
modulatory composition is flowed from the target source into contact with the first
composition.

25   72.   The device of claim 58 wherein the membrane is a component of an
intact cell.
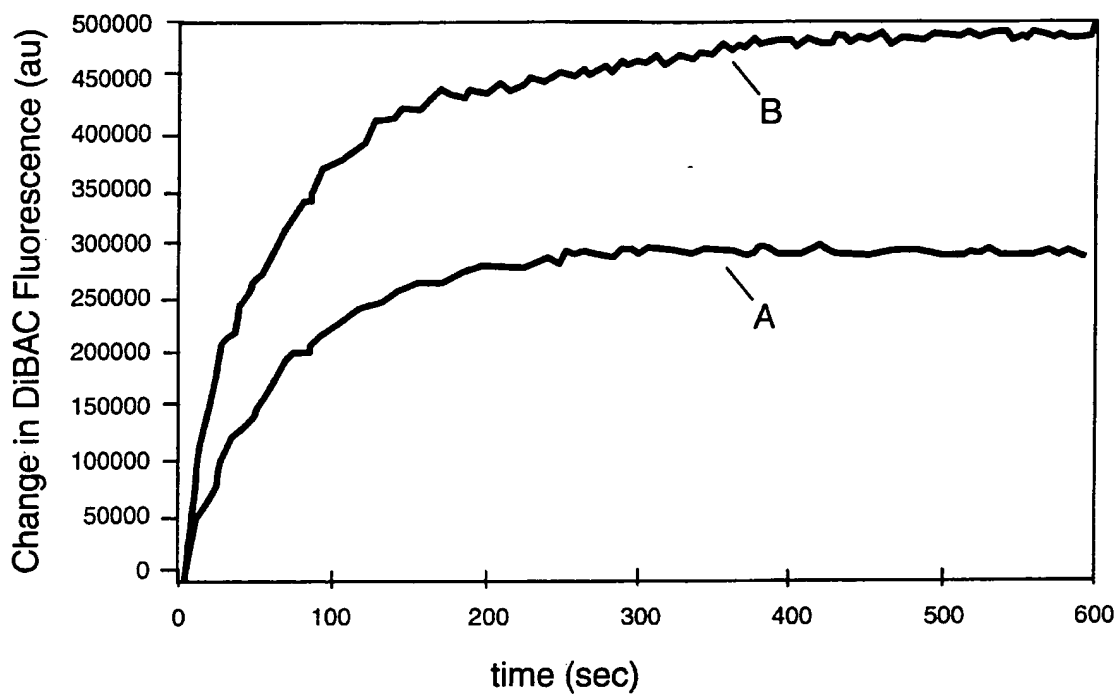
Fig. 1
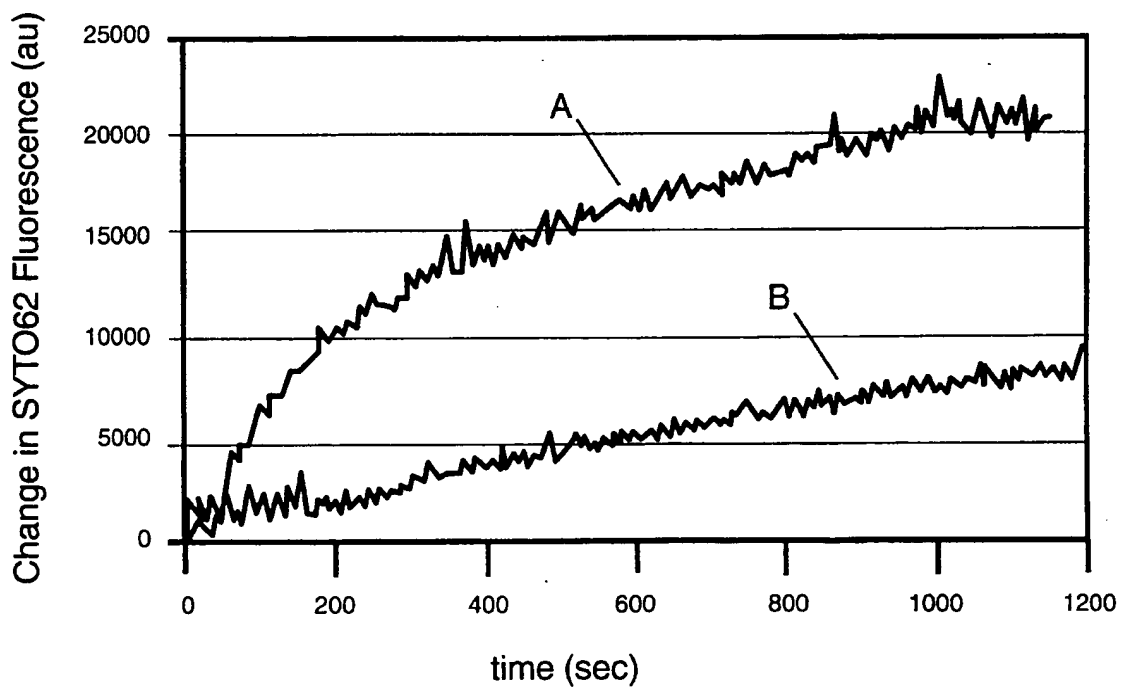
Fig. 2
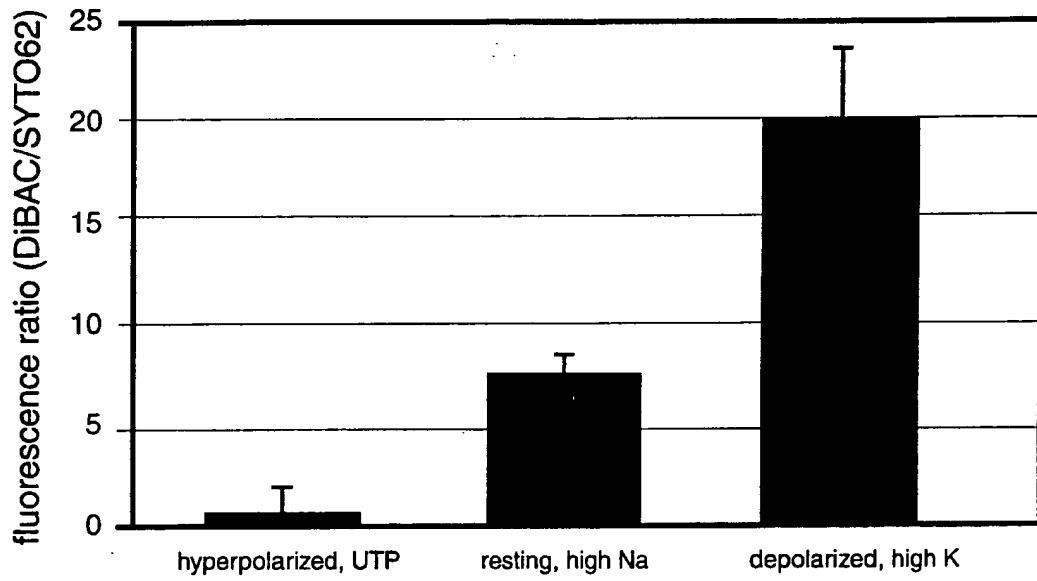
Fig. 3A

Fig. 3B

Fig. 4
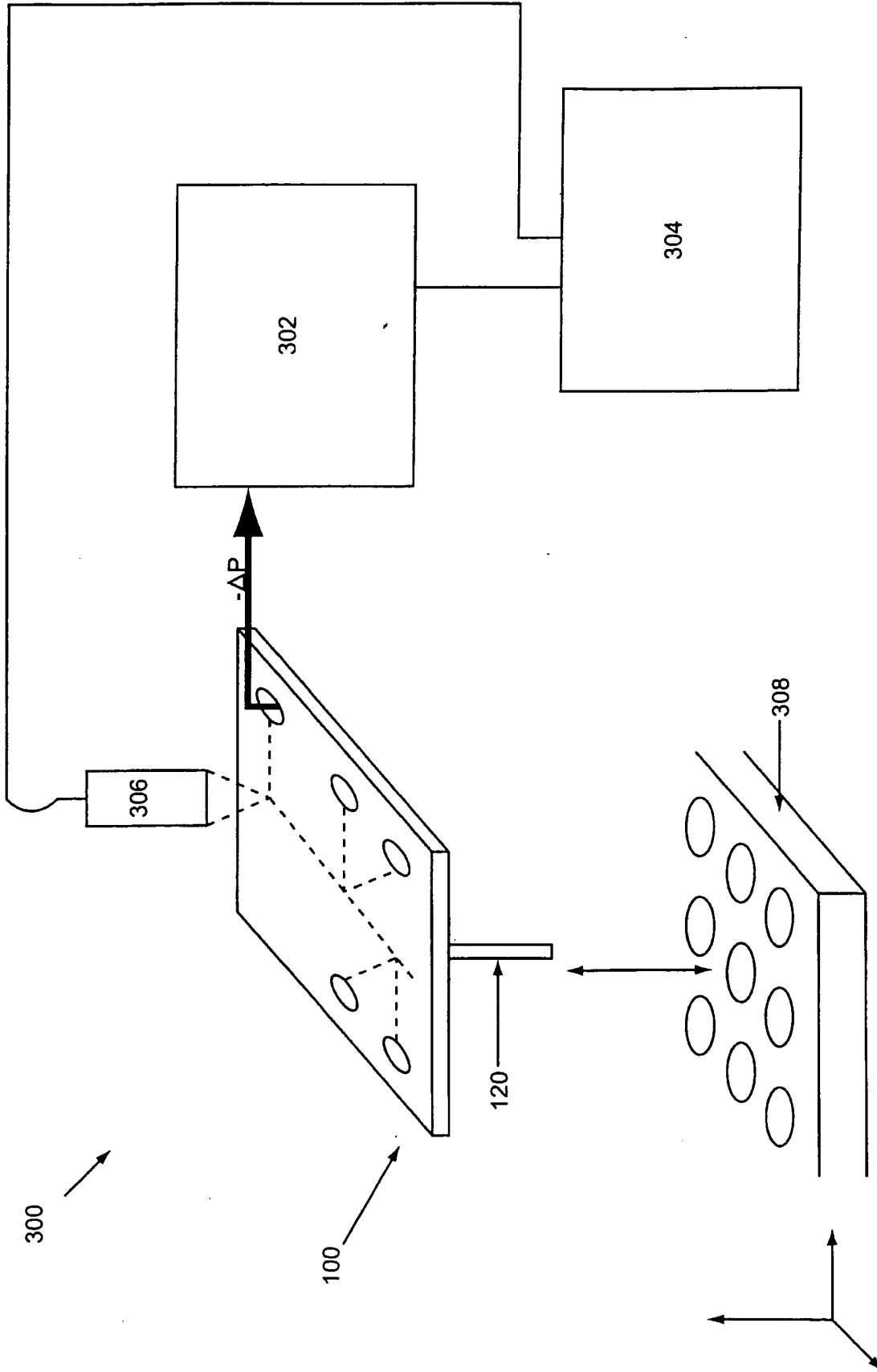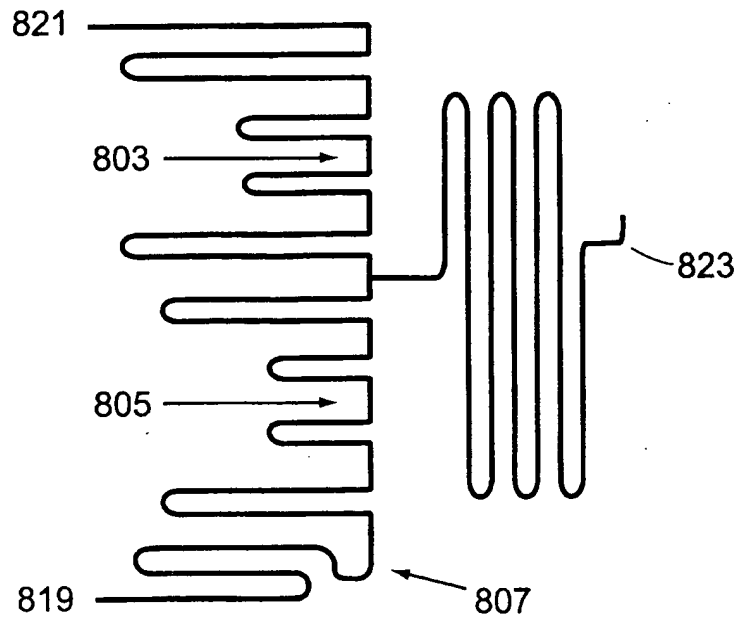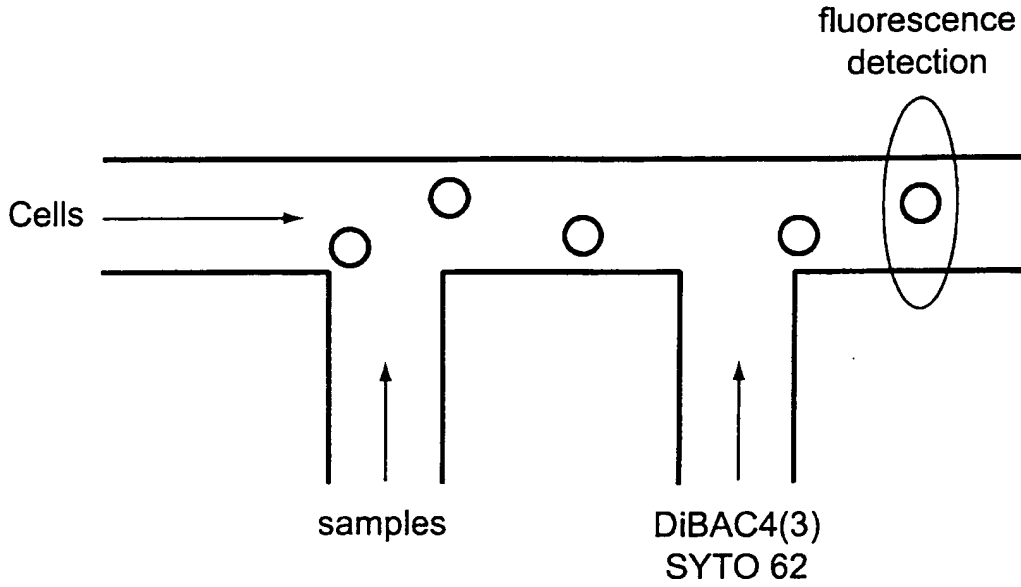

Fig. 5

Fig. 6A

Fig. 6B

Fig. 6C

Fig. 7

Fig. 8A

Fig. 8B

fluorescence
detection

Cells →

samples   DiBAC4(3)
                 SYTO 62

Fig. 9A

Fluorescence (arbitary units)

buffer   quinidine   UTP
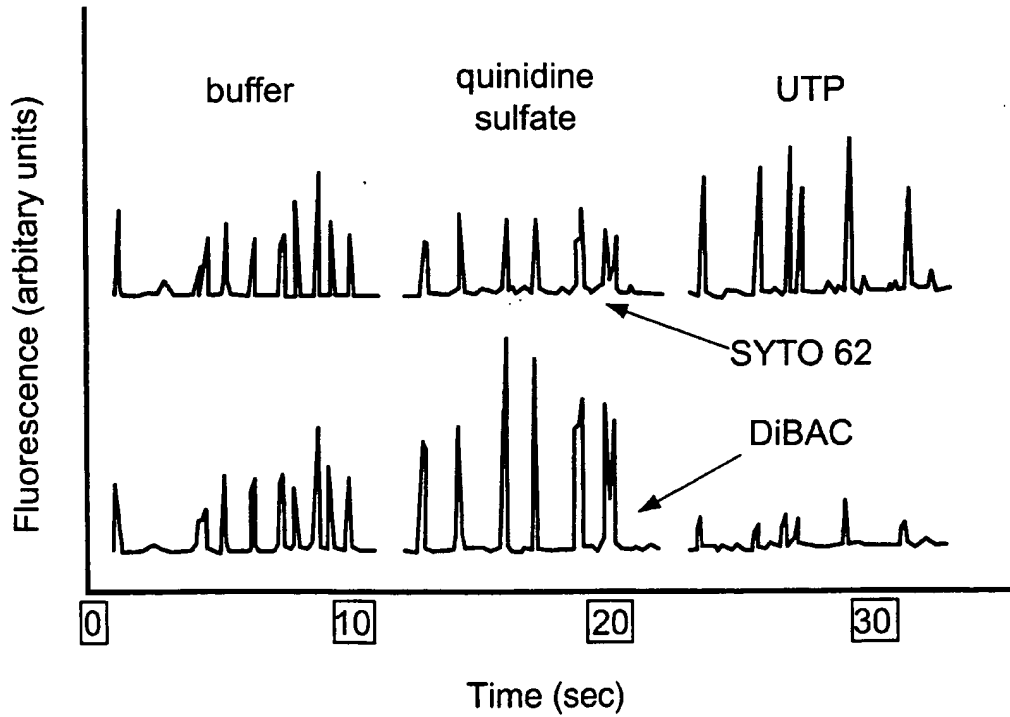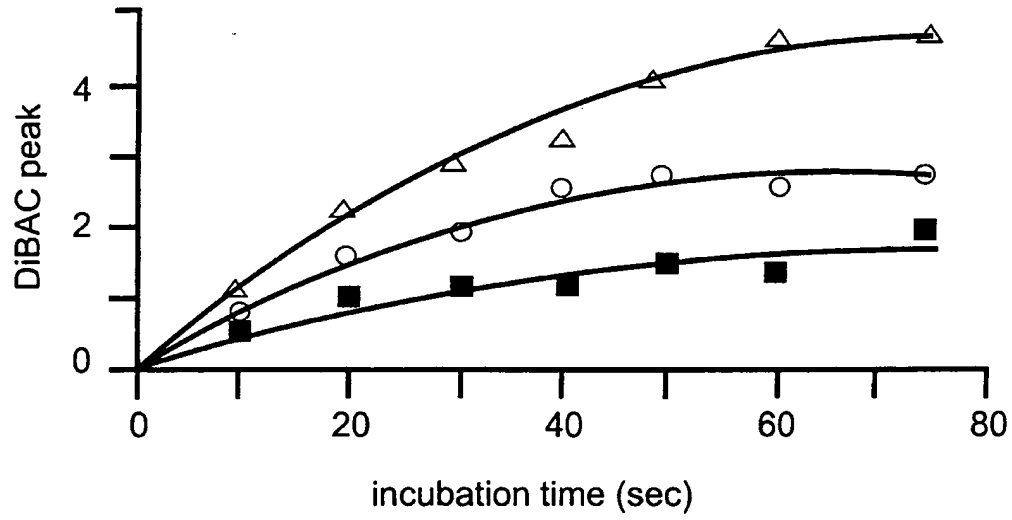   sulfate

SYTO 62

DiBAC
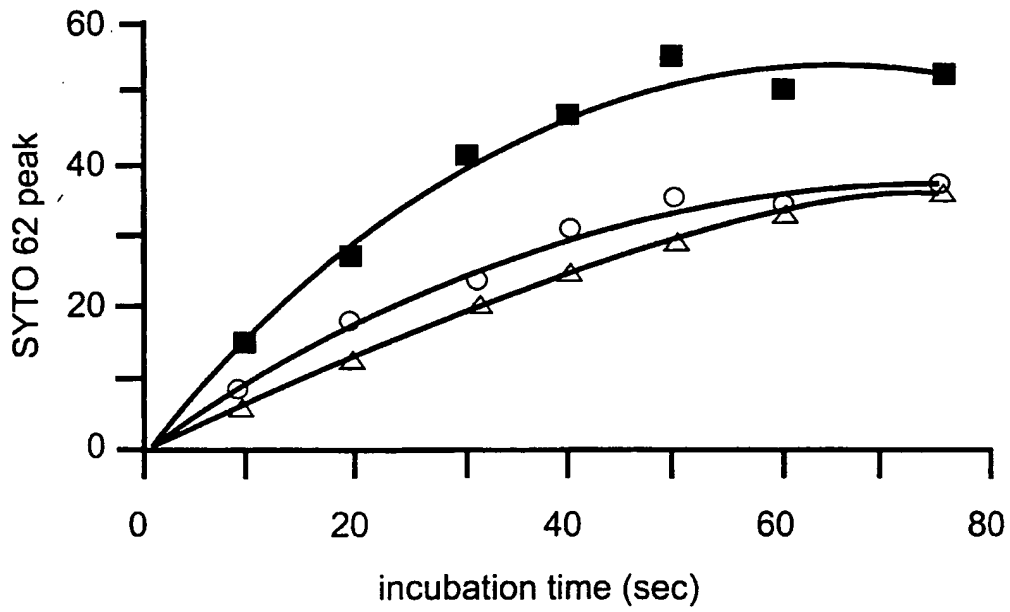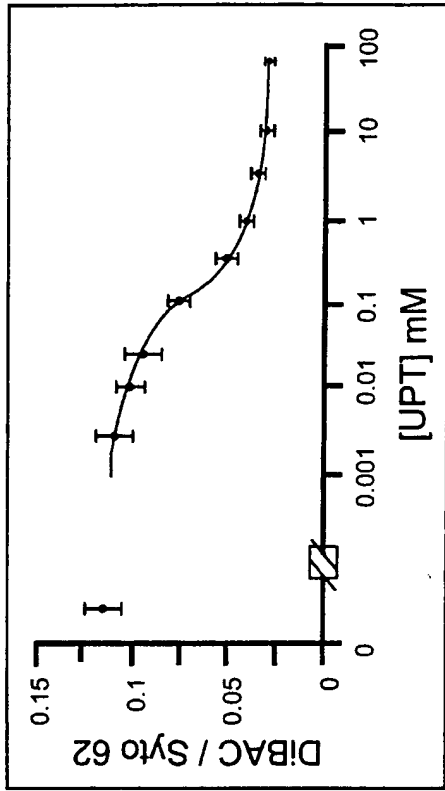
0   10   20   30

Time (sec)
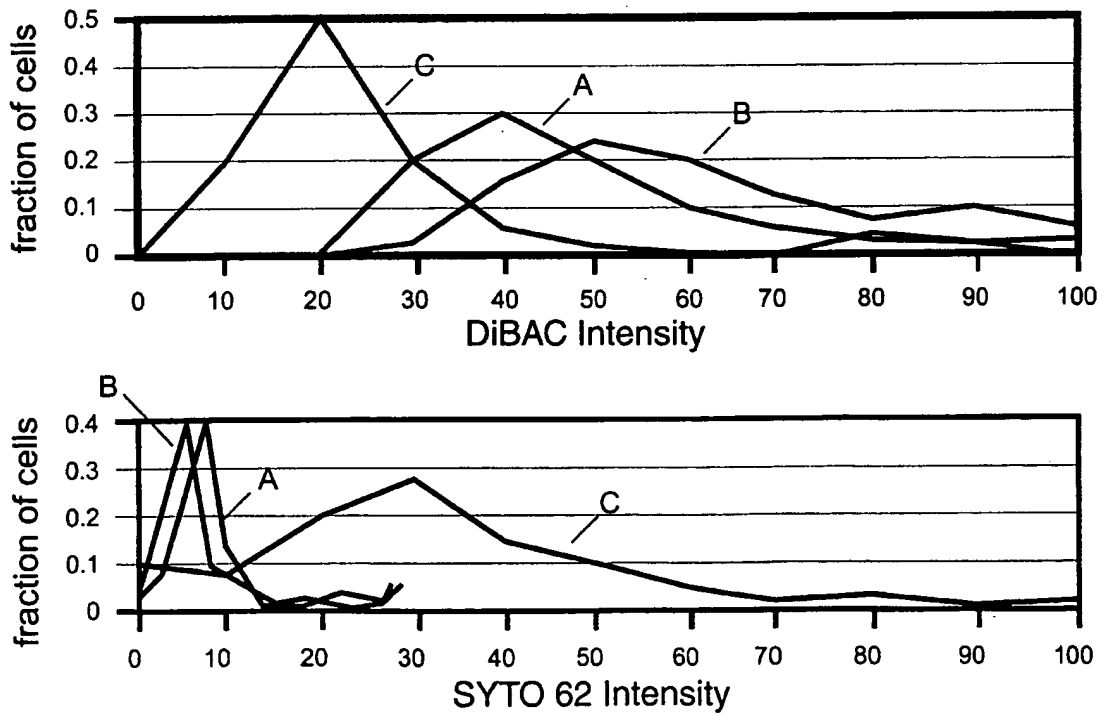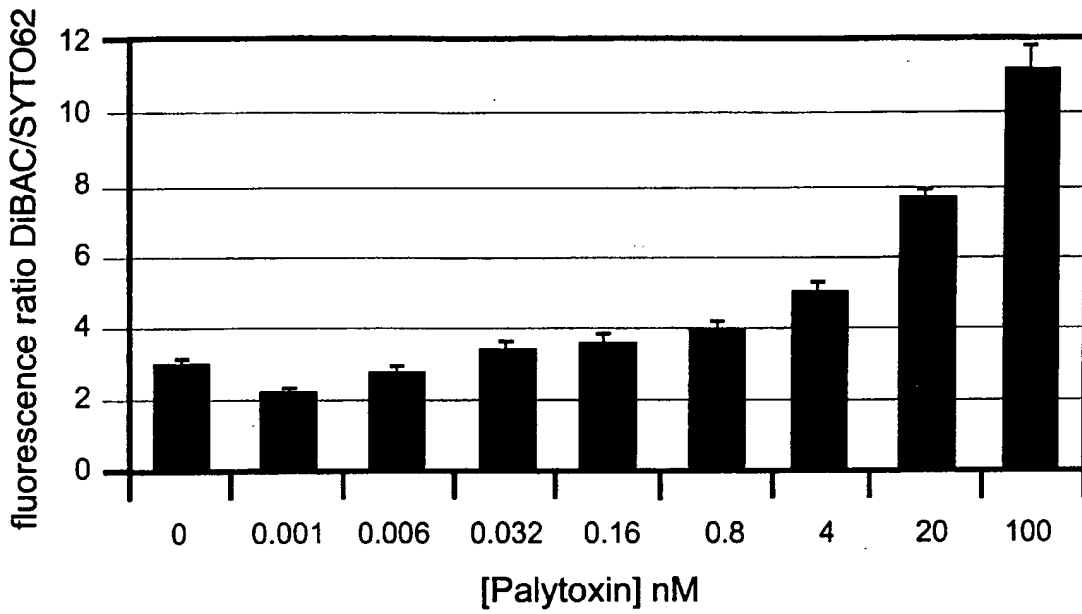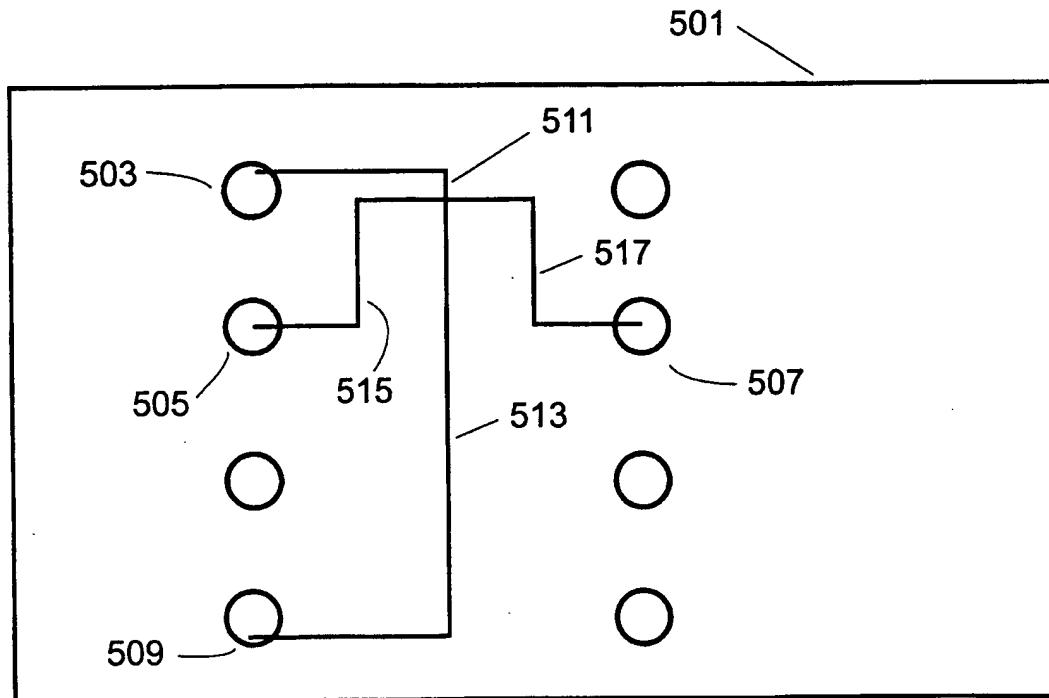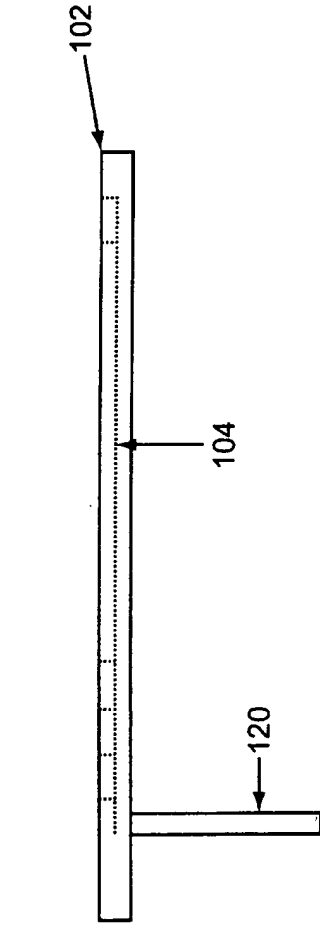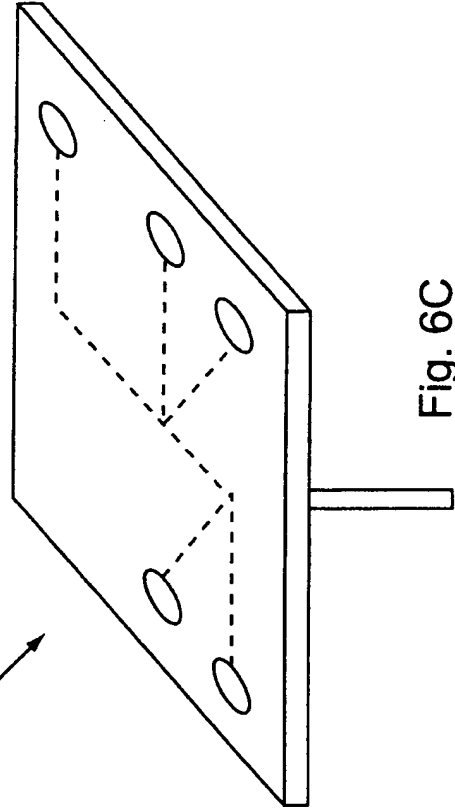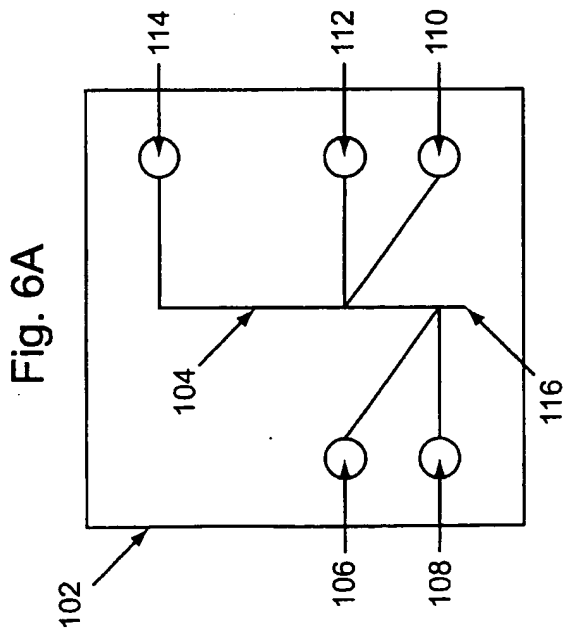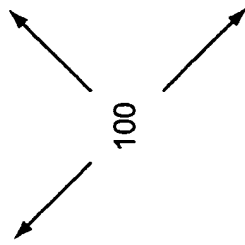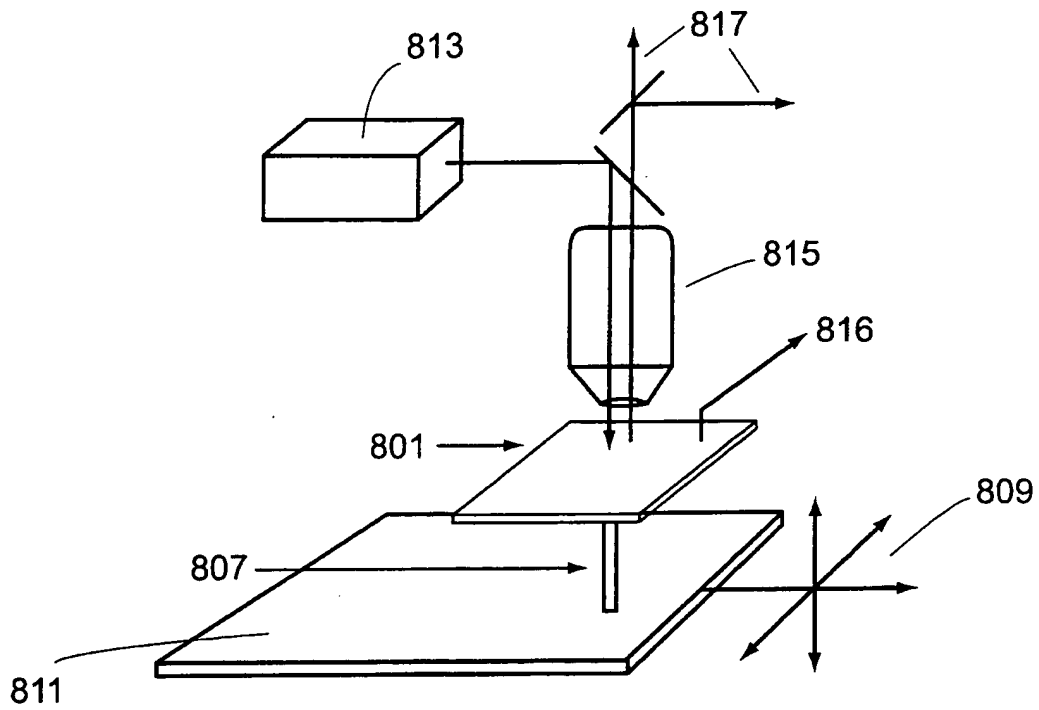
Fig. 9B

Fig. 9C

Fig. 9D

Fig. 10B
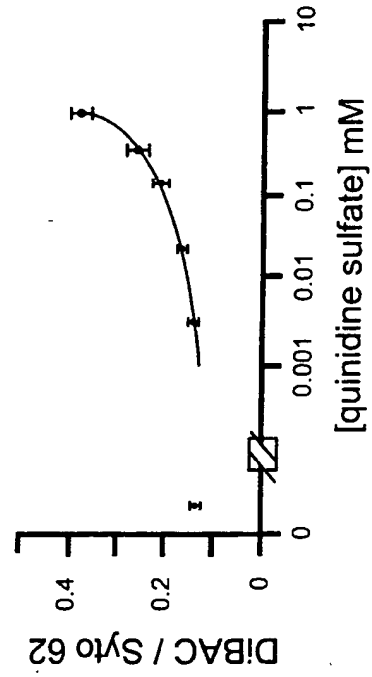
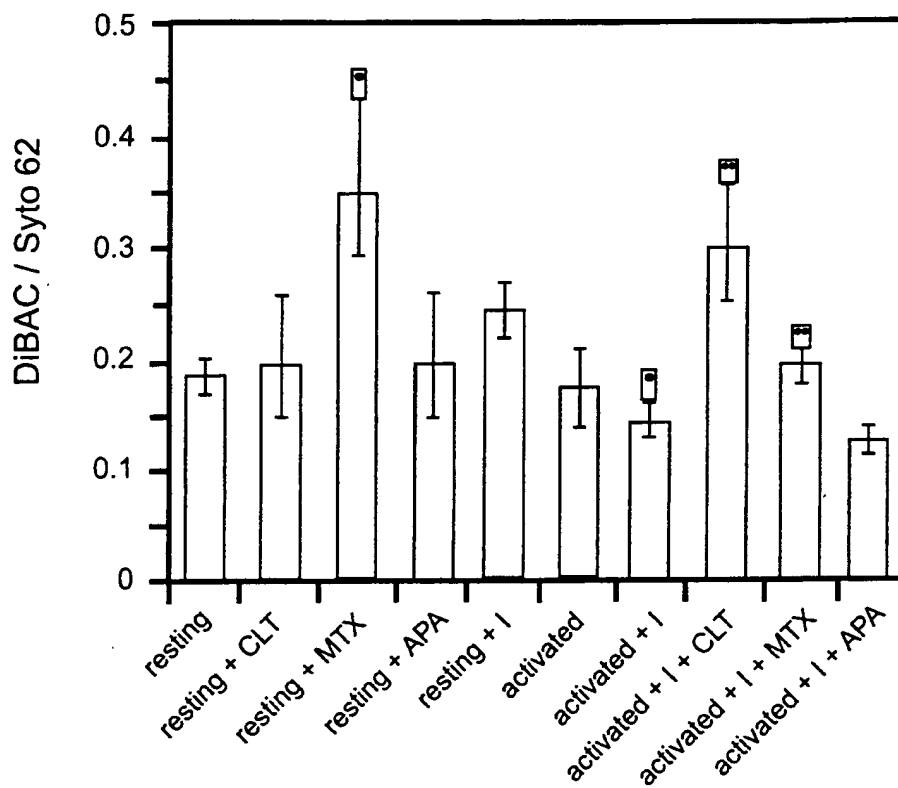Fig. 10C
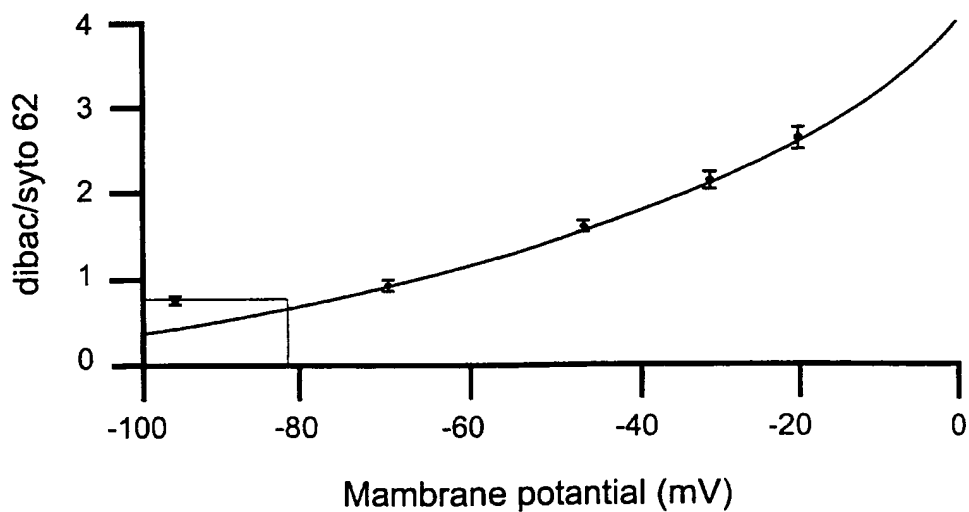
Fig. 10A

Fig. 11

Mambrane potantial (mV)
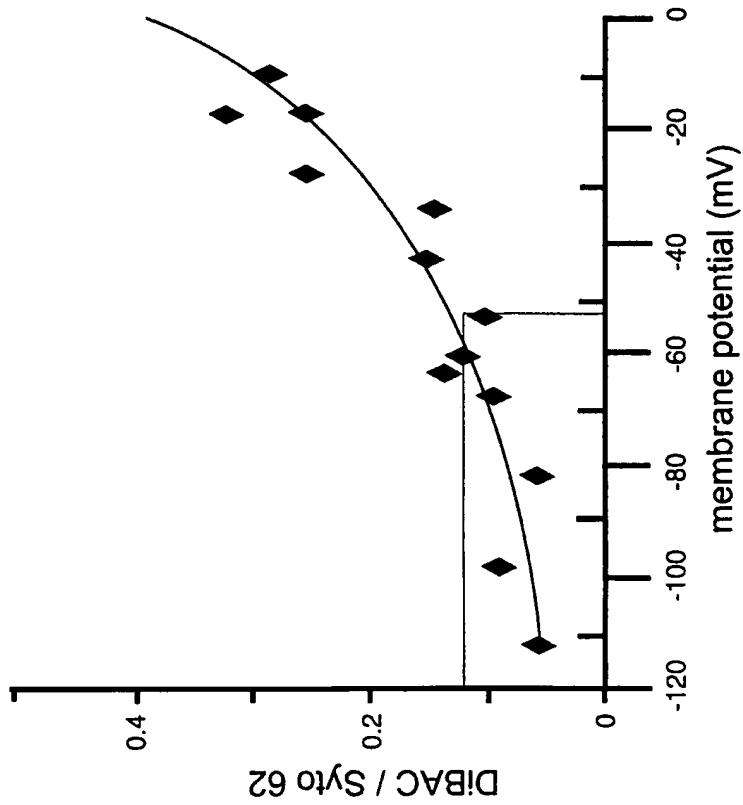
Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/27659

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 13/00; C12Q 1/02; G01N 1/30, 15/06

US CL : 435/40.5, 40.52, 173.1, 173.5, 173.6; 422/68.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/40.5, 40.52, 173.1, 173.5, 173.6; 422/68.1; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,874,668 A (XU et al) 23 February 1999 (23.02.1999), columns 9-10.	6-72
Y	US 5,661,035 A (TSIEN et al) 26 August 1997 (26.08.1997), columns 2, 4 and 12.	1-5, 7-72
Y	US 5,239,998 A (KRAUTHAMER) 31 August 1993 (31.08.1993), see entire reference.	13-41
Y	US 4,762,799 A (SEITZ et al) 09 August 1988 (09.08.1988), columns 3-4.	1-12
A	US 5,124,141 A (MAKLER) 23 June 1992 (23.06.1992), columns 3 and 6.	3, 4, 34
A	US 4,741,898 A (MALLIK et al) 03 May 1988 (03.05.1988), columns 1-2.	1-5
A,P	US 5,995,871 A (KNISLEY) 30 November 1999 (30.11.1999), see entire reference.	1-72
Y, P	US 6,107,066 A (TSIEN et al) 22 August 2000 (22.08.2000), see entire reference.	1-72

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 December 2000 (27.12.2000)	**29 JAN 2001**
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized Officer Gary Jones Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)